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BOOK REVIEW


As part of a series detailing major branches of bioprocess technology, this text covers fermentation process development with some emphasis on manipulating the biology of the organism to improve the economics of production. Broken up into six chapters, the book is arranged by organism with separate chapters covering actinomycetes, bacilli, coryneform bacteria, industrial fungi, mammalian cells and yeasts. Each production with some emphasis on detailing the role of media development to improve production and facilitate downstream processing.

The emphasis in each chapter is on a review of the practical aspects of developing fermentation processes for industrial applications. Fundamentals of strain selection, assay development and media selection are particularly well illustrated in Chapter 2 covering fermentation of Bacilli. Genetic engineering, while not the primary focus of this text, is put into focus in the chapters covering actinomycetes, bacilli, coryneform bacteria and yeasts. Chapter 5 on using mammalian cells to produce products represents a particularly useful review of mammalian cell culture technology with a useful discussion of regulatory considerations. The chapter on Coryneform Bacteria includes a good review of amino acid and nucleotide production by this group of microorganisms.

Fermentation Process Development of Industrial Organisms is meant to show the interaction between the art and science of fermentation process development research. As such, it is particularly useful to those acting at the interface between geneticists and production engineers. At the same time, it serves to help the biologist and engineer better understand the needs and capabilities of the others' discipline.

NOEL G. RUDIE
EFFECT OF HIGH-PRESSURE HOMOGENIZATION ON A STERILIZED INFANT FORMULA: MICROSTRUCTURE AND AGE GELATION

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Abstract

Age thickening and gelation of an infant formula was induced by applying high pressure homogenization prior to in-can sterilization. The initial viscosity of the preparation increased with increasing homogenization pressure. Thickening rate upon storage, as monitored by viscosity changes, was also proportional to the pressure applied during homogenization. Optical and electron micrographs of 6 month-old samples showed evidence of aggregation. The effect of dissociating agents on the viscosity and microstructure of these samples suggested a contribution of hydrogen bonds and calcium bridges to the gel integrity. The alteration of the mineral balance of aged samples also indicated a gel strengthening effect by the colloidal calcium phosphate.

Initial paper received December 6, 1989
Manuscript received March 24, 1990
Direct inquiries to Y. Pouliot
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Keywords: age gelation, age thickening, sterilization, infant formula, storage, homogenization, dissociation, viscosity, pH, transmission electron microscopy.

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Introduction

In the U.S., sterilized liquid infant formulas (ready to feed and concentrates) are more popular than the corresponding powdered product (McDermott, 1987). The shelf-life of these products can however be limited by the occurrence of fat separation during storage. Therefore, the homogenization treatment must be designed carefully in order to prevent this defect.

Homogenization is generally applied to fat containing dairy products in order to reduce the diameter of fat globules. This physical treatment reduces creaming and coalescence of the dispersed fat phase. The pressures usually applied range from 3.5 to 35 MPa, and are chosen according to several parameters: type of valve, number of stages, temperature and physico-chemical properties of the emulsion and the desired product's characteristics.

The age gelation of UHT (ultra-high temperature) and of HTST (high temperature-short time) sterilized milks have been reviewed by Harwalkar (1982). This storage defect is characterized by an increase of viscosity (age thickening) followed by the formation of a gel, usually free from syneresis, appearing after approximately three months. Age gelation has been observed in UHT, HTST sterilized milk and in-can sterilized concentrated milk. Two distinct hypotheses based on proteolytic activity and physico-chemical destabilisation have been proposed to explain the age gelation mechanism. However, there is still a lack of experimental evidence to support either of these hypotheses. Several factors influence age gelation of sterilized products: the processing conditions, the composition and microbiological quality of the product, the presence of additives and the storage temperature.

Among the processing conditions, homogenization has been identified as having a specific effect (Harwalkar, 1982). In fact, the sequence of processing steps is the determining factor, e.g., homogenization placed before the concentration step yields a product with reduced stability against gelation. When used together with a controlled heat treatment (holding at 94°C), allowing the development of a critical viscosity, homogenization can prevent age gelation. However,
the effect of homogenization pressure or of repeated homogenizations has never been reported.

Age gelation also occurs in sterilized infant formulas (McDermott, 1987) and the phenomenon is similar to age gelation of sterilized milks. In the present study, gelation of infant formula was induced by applying high-pressure homogenization prior to in-can sterilization. The gelation upon storage was followed using viscosity measurements. Dissociating agents were added to gelled samples in order to investigate the nature of interactions leading to the gel formation. Gel microstructure was examined using optical and transmission electron microscopy of resin-embedded samples.

Materials and methods

Preparation of infant formulas

A commercial liquid mix containing 7.2% fat, 14.0% carbohydrates, and 3.1% proteins (60% casein, 40% whey proteins) was prepared and the pH was adjusted to 7.0. A commercial double-stage homogenization (17-3.5 MPa) was applied to the product with a valve homogenizer. The pH was readjusted and then a second homogenization step was performed on some samples at pressures of 48 MPa or 77 MPa with a Microfluid laboratory scale homogenizer (Microfluidic Corp., Boston, MA USA). The mix was finally in-can sterilized (121°C-20 min). The cans were stored at 25°C for 6 months. Samples were taken after processing and after three and six months.

Viscosity measurements

Apparent viscosity of the samples was determined at 25°C using a Brookfield LV viscometer fitted with spindles, no. 3 and 4, (effective shear rate between 8 and 20 s⁻¹). Before samples were taken for analysis, the cans were agitated for 15 minutes with a magnetic stirrer in order to redisperse their contents and to ensure representative sampling.

Gel dissociation

The following dissociation agents were used: EDTA (ethylenediamine tetraacetate) (30 mM), urea (4 M) and β-mercaptoethanol (40 mM). The agents were added directly to the samples up to the selected concentration, the samples were agitated for 60 min before their viscosity was measured. After EDTA additions the pH was adjusted back to 6.8 using 0.1N NaOH, before viscosity measurement. 0.1N HCl and 0.1N NaOH, or Na₃H₂PO₄ and Na₂HPO₄, were added to the samples in order to study the effect of pH changes on the product's viscosity.

The results of viscosity measurements on dissociated gels are reported as dimensionless relative viscosity. This term expresses the ratio between the viscosity of samples treated with dissociating agents and the viscosity of untreated samples. This expression has been chosen to better compare the effect of the various dissociating agents.

Microscopy

Sample preparation

The samples were encapsulated in agar according to Salyaveev (1968), fixed at 4°C in buffered-glutaraldehyde (Sorensen buffer, 0.1M) at pH 6.8 for 16 h, washed in phosphate buffer for 4 h and post-fixed in 1% Oso₄ for 2 h. Dehydration was done in increasing concentrations of ethanol (30% to 100%). The capsules were impregnated overnight in a dimethylaminomethyl phenol (DMP 30)-catalysed resin mixture, and were thereafter embedded in Epon 812 for sectioning. Ultrathin preparations were made using a LKB 8600 Ultratome III (LKB-Produkter AB, Bromma, Sweden) microtome. Optical microscopy Sections of 2 μm thick were stained in 0.1% toluidine. Microscopic examination was done using a photomicroscope (Zeiss 63500) with phase contrast illumination at 80X magnification. Micrographs were taken on Panatomic-X, 32 ASA, Kodak film. Transmission electron microscopy (TEM) Sections of 80 nm thick were double-stained with 2% uranyle acetate and lead citrate according to Reynolds (1963). The examination was done using a Jeol 1200 EX transmission electron microscope, operating at 80 kV, at 8000 X magnification. Fat globule size determination Average diameters of fat particles have been measured from enlarged electron micrographs. We calculated the volume/surface average diameter \( (d_{v/s}) \) which is obtained from the following equation:

\[
\frac{d_{v/s}}{d_i} = \frac{4}{\pi} \int \frac{d^3}{d^2} \frac{1}{4}
\]

where \( d_i \) is the fat particle diameter measured on the micrograph and \( (4/\pi) \) a correction factor to take into account the random fracture plane of fat particles during sample preparation (Bird and Stainsby, 1974). The volume/surface diameter is equivalent to the particle diameter of a monodisperse emulsion having the same oil phase volume and the same interfacial area.

Results and discussion

Viscosity of homogenized infant formulas

Homogenization reduces fat particle size and creates new oil/water interface according to the pressure used (Mulder and Walstra, 1974). During the homogenization process, the newly formed interface is rapidly covered with amphiphilic material (mainly proteins) (Cortwijn and Walstra, 1979). We observed that the initial viscosity of infant formula increased with the pressure applied (table 1). As a first approximation, the increased viscosity resulting from homogenization could be attributed to the newly formed membrane which increases the effective dispersed phase fraction of the emulsion (Bird and Stainsby, 1974). High pressures generate larger interface which allow more proteins to be integrated in the dispersed phase of the emulsion.

However, it is unlikely that the increase of dispersed phase fraction upon homogenization is the only factor responsible for viscosity of infant formula. Other variables such as polydispersibility, degree of aggregation, particle size, shape, deformability, and viscoelastic properties of the membranes have been shown to affect the viscosity of suspensions (Dickinson and Stainsby, 1982). One should consider the effect of homogenization on...
Age gelation of sterilized infant formula

Table 1 Effect of homogenization pressure on the viscosity of homogenized infant formulas immediately after processing.

<table>
<thead>
<tr>
<th>Homogenization pressure (MPa)</th>
<th>Viscosity (mPa.s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-3.5 (double stage)</td>
<td>10</td>
</tr>
<tr>
<td>19</td>
<td>15</td>
</tr>
<tr>
<td>48</td>
<td>37</td>
</tr>
<tr>
<td>77</td>
<td>46</td>
</tr>
</tbody>
</table>

these parameters to better understand the phenomenon.

Effect of high-pressure homogenization. The viscosity changes upon storage have been measured after three and six months (figure 1). The formula homogenized at high pressures (48 MPa and 77 MPa) showed evidence of age thickening and gelation. The rate of change of viscosity was related to the pressure used to homogenize the product. It appears that high-pressure homogenization accelerates the phenomenon of age gelation according to the pressure used. This trend has also been observed for UHT and sterilized concentrated milk (Harwalkar, 1982).

![Figure 1. Viscosity changes (over 6 months at 25°C) of sterilized infant formula homogenized at 17-3.5 MPa (o), 48 MPa (●) or 77 MPa (Δ).](image1)

Age thickening and gel formation are related to the aggregation of protein material in the product. Therefore, according to its effect on these phenomena, homogenization is believed to alter the interaction potential of proteins. However, it has been shown that even the large casein micelles are too small to be directly affected by homogenization (Walstra, 1980). It is then likely that protein adsorption and spreading onto fat droplets, which occur during homogenization, are responsible for increased sensitivity to aggregation. Unfolding of proteins might expose new regions of the structure that promote interactions. Absolom et. al. (1987) studied the surface properties of protein-coated polymers and reported an important drift of protein surface energy upon adsorption. Assuming that proteins adsorbed onto fat droplets are more sensitive to aggregation than non-adsorbed proteins, one could relate the aggregation potential of an emulsion to the proportion of the total proteins adsorbed onto the fat droplets.

Considering the fat and protein content of infant formula, one could approximate the effect of reducing fat droplets size on the proportion of adsorbed and non-adsorbed protein. For that purpose, we assumed a constant protein load of fat droplets of 10 mg/m² (Melsan and Walstra, 1989). There is however limitation to this assumption. First it has been shown, in emulsions containing casein micelles, that the protein load tends to increase with decreasing particle size (Walstra and Oortwijn, 1982), and second, for very fine particle dispersion, the lack of protein could limit the amount adsorbed which in turn would limit the protein load. Despite these limitations, the calculation has been done and the results are presented on figure 2. Decreasing particle size of the fat droplets induces an approximately hyperbolic increase of the proportion of adsorbed proteins. Inversely, the amount of free proteins is reduced. Average particle size was determined on our samples from the electron micrographs. According to these calculations, the average diameter (volume/surface) of low (17-3.5 MPa) and high (77 MPa) pressure homogenized samples were respectively 0.71 and 0.25 μm. In relation with figure 2, the high pressure conditions would increase the proportion of adsorbed protein from 19 to 55%. Therefore, it is believed that the large amount of proteins sensitized to aggregation (via adsorption onto fat droplets) could explain the rapid viscosity increase during the storage of these samples.

![Figure 2. Distribution of proteins in infant formula as a function of the mean particle size of the fat dispersion.](image2)

The inclusion of small oil droplets into the protein matrix could also be responsible for accelerated age gelation of high pressure homogenized samples. Tiny oil droplets, presumably covered with proteins, participate in gel formation and increase the volume fraction of the gel matrix.
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Figure 3. Microstructure of sterilized infant formula after 3 months storage: (A) commercial sample under optical microscope; (B) 77 MPa-homogenized sample under optical microscope; (C) TEM micrograph of commercial sample; (D) TEM micrograph of 77 MPa-homogenized sample. Bar = 100 μm for A and B, 1 μm for C and D.

Integration of tightly attached fat in protein clusters has been shown by Geyer and Kessler (1989).

As seen from figure 3, the coarse structure of products homogenized at low and high pressure can be observed using optical microscopy as the microstructural details may be better seen on the electron micrographs. We observed from optical micrographs that the gelled sample (figure 3C) showed uneven morphology composed of large aggregates. The aggregates in the ungelld sample (figure 3A) are much smaller and uniformly distributed. The electron micrographs revealed the presence of both fat globules (fg) and casein micelles (cm). Large globules (up to 1 μm) have been found in the commercial sample (figure 3C). The high pressure homogenized sample (figure 3D) gelled and showed some "chain-like" arrangements composed of fat globules and casein micelles. The large colloidal surface associated with the size of fat droplets might have promoted associations between colloidal particles. The absence of free casein micelles is also noticeable in that sample. Again, considering the average size of fat particles (0.25 μm), most of the micelles are believed to be adsorbed on fat surface. Such interactions between fat particles and casein micelles could be suspected from the work of Wilson et al. (1963) on gelled concentrated milks.

Breakdown of gel matrix

Effect of dissociating agents. Different dissociating agents have been tested in order to break down the gel matrix obtained after storage. The reagents have been chosen in order to determine the types of interactions responsible for gel formation. Selective dissociating agents were: EDTA (for calcium bridges), urea (for H-bonding) and β-mercaptoethanol (for S-S bonds).

From viscosity data (table 2), it was observed that EDTA increased the viscosity of the sample. Calcium chelation may have caused some swelling of the casein micelles, which would explain the viscosity increment despite the dissociating action of EDTA. The two other dissociating agents (urea and β-mercaptoethanol) produced a decrease in viscosity. When a binary mixture of dissociating agents was used, a synergistic effect was observed for those containing urea. This effect suggests that not all H-bonds are accessible to urea. Calcium bridges or S-S bonds need to be disrupted to allow an extensive action of urea. The very low viscosity of the EDTA-Urea treated samples emphasizes the importance of H-bonds and calcium bridges in the gel structure.

Table 2 Effect of dissociating agents on the relative viscosity of infant formula gels.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative viscosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated (gelled)</td>
<td>1.00</td>
</tr>
<tr>
<td>Mercaptoethanol (40 mM)</td>
<td>0.52</td>
</tr>
<tr>
<td>Urea (4 M)</td>
<td>0.46</td>
</tr>
<tr>
<td>EDTA (30 mM)</td>
<td>1.31</td>
</tr>
<tr>
<td>Mercapto. + Urea</td>
<td>0.37</td>
</tr>
<tr>
<td>Mercapto. + EDTA</td>
<td>0.82</td>
</tr>
<tr>
<td>Urea + EDTA</td>
<td>0.06</td>
</tr>
</tbody>
</table>

The optical micrographs provided some confirmations of the previous findings using viscosity measurements. Samples treated with EDTA exhibited some gel disaggregation (figure 4B). The combination EDTA-Urea (figure 4D) produced the finest dispersion, suggesting an extensive dispersion of the gel. However, when urea was used alone (figure 4A) or with β-mercaptoethanol (figure 4E), no evidence of disaggregation was found. When β-mercaptoethanol was used alone (figure 4C) or with EDTA (figure 4F), large
Age gelation of sterilized infant formula

Figures 4 and 5. Microstructure (optical micrograph) of infant formula gels upon added dissociating agents: (A) Urea 4 M; (B) EDTA (30 mM); (C) B-Mercaptoethanol (40 mM); (D) Urea (4 M) + EDTA (30 mM); (E) Urea (4 M) + B-Mercaptoethanol (40 mM); (F) B-Mercaptoethanol (40 mM) + EDTA (30 mM). Bar = 100 μm.

Aggregates were found, questioning the implication of S-S bonds in gel matrix. However, Wilson et al. (1963) observed a quick release of fat globules from the protein-fat aggregates in the presence of reducing agents (Na₂SO₃ and ascorbic acid). According to the authors, this effect was due to the occurrence of disulfide bonds in the gel structure, surrounding the fat particles.

The electron micrographs (Figure 5) provided limited information on the gel structure since the occurrence of "chain-like" structure was found on all micrographs. However, one should remember that TEM is not the best suited method to reveal associations between particles since only the associations in the plane of the observation can be observed (Kalab et al., 1976). None of the tested dissociating agents affected the size of fat particles.

Effect of pH changes. Since the effectiveness of the dissociating agents used in our study was due to their action on the proteins, it was thought that other variables affecting the proteins would also affect the gels. So the pH was modified using separately NaOH and HCl, or NaH₂PO₄ and Na₂HPO₄, and the viscosity was monitored over the 6.5-7.5 pH range.
In fact, altering the pH of the gelled samples with NaOH or HCl had an important effect on viscosity (Figure 6). This phenomenon is believed to be related to the effect of pH on the casein micelle’s colloidal calcium phosphate (Pyne and McGann, 1960) which might contribute to gel formation. Increasing the pH, presumably increased the colloidal forms of calcium phosphate, and thus, increased the viscosity of the sample. Conversely, decreasing pH solubilized some colloidal calcium phosphate and reduced sample viscosity. When the pH was altered using sodium phosphate salts, the excess of phosphate ions possibly exceeded the solubility product of calcium phosphate. Thus, higher proportions of colloidal calcium phosphate were expected than with HCl or NaOH at any pH. As seen from Figure 6, samples having had their pH altered with sodium phosphate salts showed higher solubility product of calcium phosphate. Thus, decreasing pH solubilized some colloidal calcium phosphate to the age gelation of infant formula. The results of this study suggest that high pressure homogenization removes casein micelles from the serum phase as they form a tightly bonded fat protein complex. Spreading of casein on fat particles is believed to increase their interaction potential and their availability for association, which in turn explains the acceleration of age gelation phenomenon. Breakdown of gel matrix when using dissociating agents suggests that hydrogen bonds and calcium bridges contribute to the gelation phenomenon upon storage. Furthermore, the equilibrium between the colloidal and soluble forms of calcium phosphate presumably has a determining effect on the gel strength in the process of age gelation of sterilized infant formula.

**Figure 6.** Effect of pH changes on the relative viscosity of infant formula gels, using HCl and NaOH (o), or NaH₂PO₄-Na₂HPO₄ (Δ).

**Conclusion**

The results of this study suggest that high pressure homogenization removes casein micelles from the serum phase as they form a tightly bonded fat protein complex. Spreading of casein on fat particles is believed to increase their interaction potential and their availability for association, which in turn explains the acceleration of age gelation phenomenon. Breakdown of gel matrix when using dissociating agents suggests that hydrogen bonds and calcium bridges contribute to the gelation phenomenon upon storage. Furthermore, the equilibrium between the colloidal and soluble forms of calcium phosphate presumably has a determining effect on the gel strength in the process of age gelation of sterilized infant formula.

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**References**


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Age gelation of sterilized infant formula

Discussion with reviewers

P. Waiistra: The 15 min agitation using a magnetic stirrer may considerably lower the apparent viscosity, or even redisperse the gel, so that the results of the viscosity test may be rather meaningless. Please comment.

Authors: We agree with the idea that mechanical stress reduced the apparent viscosity of our samples. However, the gravitational separation of the product upon storage forced us to re-establish the homogeneity before sampling. Another approach would have been to store the products in a device which maintains slow rotation in order to avoid gravitational effects. However, even then, the transfer of the product from the can to the rheometer cell would have induced some gel disruption which is still difficult to control. For this reason, we chose the first approach which consisted in applying a controlled mechanical stress prior to the viscosity measurement. The product's viscosity was then related to the mechanical resistance of the gel which depends on the strength of interactions within the matrix.

P. Waiistra: I thought it was fairly clear that one mechanism (proteolysis) is responsible for age gelation in UHT un-concentrated milk products, and the other one (physico-chemical) normally in evaporated milk?

Authors: From the evidences presented by Harwalkar (1982), we believe that it is not clear whether the two hypotheses can be associated with one or the other type of sterilized products (see pages 260 to 264). In this review, it is demonstrated that age gelation can occur in UHT un-concentrated milk products showing no proteolysis. The age gelation phenomenon can be seen as the result of more than one mechanism.

B.E. Brooker: When using the agar tube technique with your material, are milk solids uniformly distributed along the tube after embedding or are there local concentrations resulting from the removal of water during dehydration?

Authors: We have not observed such concentration gradient in our preparations. It seemed that the glutaraldehyde fixation maintained an homogeneous solids distribution within the sample. From the observation of larger embedded sections under optical microscope, we could not notice differences in structure between the center and the edge of the capsule. Jewell (1981) used successfully the agar tube technique for fruit juices, which are less viscous and contain much less protein than our product. These points suggest that the solid distribution in our samples was not affected by the preparation technique.

D.N. Holcomb: While the analytical composition of the liquid mix is given, details of the ingredients are not given. Without such details, a reader could not duplicate the experiment.

P. Waiistra: Was the milk pre-heated? Is the casein present in micellar form?

B.E. Brooker: Since the observations made in this paper hinge on the chemical composition of the milk, more details of the preparation of the infant formula should be given. Thus, is all of the fat butterfat? Is the 14% carbohydrates predominantly lactose? Was the mixture prepared from reconstituted milk powder?

Authors: As aforementioned, the liquid mix was a commercial product of which detailed formulation and process could not be divulged. However, we know that a pre-heating treatment was applied to the product but we could not have details about the exact conditions. Also, from a partial list of ingredients, we know that this product contains skim milk solids (thus, the caseins were added in micellar form), lactose, soya and coconut oil, mono- and di-glycerides, lecithin from soya and carrageenin.

M. Ruegg: The role of whey proteins have not been discussed enough. The high temperatures applied to the samples suggest a complete denaturation of whey proteins and it is known that the hydrophobic whey proteins are adsorbed on both, casein micelles and fat globules. The authors should comment on this.

Authors: For the purpose of this study, we have considered the protein fraction of infant formula as a whole. However, we agree with the reviewer that this type of protein could affect the viscosity change upon storage. During the sterilization treatment, whey proteins are believed to adsorb onto fat droplets and casein micelles, changing their surface properties. It is also believed that the heat treatments alter whey protein molecules already adsorbed onto fat droplets. Those changes could affect the gelation kinetics of infant formula. However, since the amount of whey proteins and the severity of heat treatment were the same for all samples, it is not possible from our data to further discuss those points.

M. Ruegg: The high temperatures and pressures applied to the infant formulas cause denaturation and precipitation of whey proteins and possibly alteration of caseins. What is the authors opinion about the application of chromatographic methods to investigate the state and role of the various proteins on the fat globule surface?

Authors: The homogenization pressure and sterilization temperature could affect the composition and surface properties of the protein layer which surrounds fat droplets. To study the fat droplet surface properties, one could use hydrophobic chromatography in the same way as used to characterize the surface properties of bacteria (Dahlback et al., 1981). However, the polydispersity of fat droplet could interfere with the method. We would also suggest the methods which have been used to characterize proteins adsorbed onto polymers, such as sedimentation volume and contact angle measurements (Absolom et al., 1987). Those approached could be adapted to study the surface properties of the protein membrane adsorbed onto fat droplets.
Y. Pouliot et. al.

Additional references


IDENTIFICATION AND CHARACTERIZATION OF COCOA SOLIDS AND MILK PROTEINS IN CHOCOLATE USING X-RAY MICROANALYSIS

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Abstract

X-ray microanalysis was used to compare the elemental composition of isolated cocoa solids with milk powder. Whereas milk powder contained similar high levels of calcium and potassium, cocoa solids were rich in potassium but contained very small amounts of calcium. This difference in elemental composition allowed the two populations of particles to be distinguished in digital X-ray maps of frozen and fractured samples of plain, milk and white chocolates examined by cryo-scanning electron microscopy. The size distributions of particles imaged in this way were determined using standard software and were used to distinguish between two white chocolates whose particle reduction (refining) parameters during processing were different.

This technique offers the possibility of studying particle interactions during processing and the influence of shape, size and spatial distribution of milk and cocoa solids on the rheology and mechanical properties of chocolate without destroying its normal structure.

Introduction

The sugars, proteins and lipids of milk ingredients used to make milk chocolate take part in complex chemical and physical interactions during processing which impart important attributes of flavour, colour and texture to the final product (Kinsella, 1970; Reimerdes & Mehrens, 1988). The milk proteins in particular possess a range of desirable properties including surface activity, modification of rheology and a marked chemical reactivity. It is apparent also that the proteins are important determinants of the structural and functional properties of chocolate which, in their turn, influence texture and flavour. Thus, for a given chocolate, changes only in the nature of the milk added can produce significant changes in the product (Reimerdes & Mehrens, 1988).

Apart from milk solids, chocolate contains other hydrophilic particles of cocoa solids (defined here as non-fat cocoa solids) and sugar crystals which, during processing, undergo marked changes and take part in important interactions that strongly influence the organoleptic properties of the chocolate (Cook, 1982). Thus, particle size distribution affects the final flavour and mouth feel (Cook, 1982) such that chocolate is perceived as coarse if more than 20% of the particles are larger than 22 μm (Rostagno, 1969). However, attempts to study the interactions of these particles, their role in the uptake of water by chocolate and the way in which their dimensions are influenced by processing parameters have been thwarted by the lack of suitable methods for discriminating between, and obtaining quantitative information from, the different populations of particles in bulk samples of intact chocolate.

Whilst it has long been possible to recognise birefringent crystals of sucrose and cocoa butter in sections of chocolate examined in polarized light (Hanssen, 1970), the ability to specifically identify cocoa solids and milk protein in a similar way but without the use of solvents and at higher resolution would be a valuable technique. An approach which requires the examination of defatted and stained sections of chocolate by light microscopy is limited by resolution and relies on non-specific staining reactions to visualise milk protein; there is also the danger

Keywords: Chocolate, X-ray microanalysis, X-ray mapping, cryo-scanning electron microscopy, cocoa solids, milk protein, plain chocolate, white chocolate, crumb chocolate, dry mix chocolate.
of altering the spatial relationships of particles by extracting the continuous fat phase with solvent (Heathcock, 1985). Procedures for the fixation, embedding and sectioning of chocolate for examination by transmission electron microscopy (TEM) are successful in identifying particles of milk powder and cocoa solids, but the specimens used are of necessity very small and the fixation time is very extended (Cruickshank, 1976; Heathcock, 1985). Although some work has been reported in which metal replicas of frozen and fractured chocolate were examined by TEM to obtain fine details of the structural relationships between these components (Buchheim & Knoop, 1971; Heathcock, 1985), again, only small samples can be used with this technique and an overall evaluation of particle size, distribution and relative position is difficult to obtain.

Cold stage scanning electron microscopy (cryo-SEM) has the great advantage that it allows the examination of frozen and fractured bulk samples of food materials which are quite stable in the electron beam and in which details of the internal structure can often be seen (Schmidt & van Hooydonk, 1980; Brooker et al., 1986, Brooker, 1988a, 1988b), but chocolate is an exception. Thus, whilst Heathcock (1985) and Sargent (1988) were able to recognise occasional particles of dried milk and sugar in bulk samples of chocolate examined in this way, the identification of all such components, including cocoa solids, exposed at the surface of the fracture cannot be done reliably using morphological criteria only. In the present study, an improved technique is described in which X-ray spectrometry is used in conjunction with cryo-SEM and in which the identification of particles is based on their elemental composition.

Materials and Methods

Chocolate solids and milk powder

The distribution of different elements in particles of spray dried skimmed milk powder and in the cocoa solids of cocoa mass was studied using energy dispersive X-ray spectrometry (see below). Cocoa mass was obtained from a commercial source and defatted using several changes of a 60:40 mixture of chloroform and methanol. Between washings with this solvent, insoluble cocoa solids were allowed to sediment and after the final wash, excess solvent was evaporated. The layer of cocoa solids produced in this way was dispersed by crushing gently with a spatula and small portions of the resulting powder were then applied as a thin layer to graphite stubs which had been smeared previously with a thin adhesive layer of carbon dag. Stubs were then coated with a conducting layer of carbon by evaporation in an NGN coating unit. Commercial spray dried skimmed milk powder was mounted on stubs and coated with carbon in the same way.

Samples were examined in a Hitachi S-570 SEM fitted with an energy dispersive X-ray spectrometer and both spectra and digital X-ray maps were obtained from them as described below. Chocolates

In order to study structural aspects of chocolates by SEM and to determine the distribution of elements in intact chocolate products, it was necessary to use cryo-SEM in conjunction with X-ray microanalysis. The application of a low temperature technique, using an EMscope SP2000 cryo-preparation unit with transfer device and microscope cold stage, ensured the stability of the specimens and the immobilization of their components during examination in the electron beam. A modified specimen holder was used (Fig. 1), unlike the standard EMscope holder, was not fitted with the shroud that was originally designed to prevent the formation of ice on the specimen during transfer between the preparation chamber and the SEM. However, under the conditions of specimen handling used in this study, experience has shown that ice does not form on the surface of specimens during transfer if sufficient care is taken.

The vacuum system of the EMscope SP2000 cryo-preparation unit was specially modified to allow the more efficient and more controlled evaporation of carbon during specimen coating. By fitting a Leybold 50 l turbomolecular pump between the work chamber and the rotary pump (Fig. 2), carbon evaporation time was reduced to a pressure better than 10⁻⁶ Torr rather than at the pressures achieved with a cryo-assisted rotary pump (10⁻²-10⁻³ Torr). Used in this way, several specimen coatings could be obtained from a single length of carbon cord.

Some preliminary studies reported by Heathcock (1985) suggested that freezing chocolate from ambient temperature produced fractures which passed only through the continuous fat phase. It was suggested that chocolate should be heated to 40°C to melt the fat phase before freezing and fracturing the specimen to reveal the internal particulate components. Under these circumstances, the plane of fracture was believed to pass around the continuous cocoa butter phase rather than through it, thus permitting the populations of particles to be seen more easily and, by inference, to produce a better X-ray signal. Preliminary observations did not support these results but, in some experiments, the X-ray spectra and maps from chocolate prepared at 40°C in this way were compared with those obtained using the standard method described below.

Generally, samples were placed in an oven at 28°C for at least 1h before use to allow intact slices of chocolate, 2 mm thick, to be cut with a razor blade without crumbling. Slices were then placed in the groove of a circular carbon insert on a pre-warmed (28°C) specimen holder and secured in place using carbon cement (Fig. 2). After freezing by plunging into nitrogen slush, the sample was transferred to the preparation chamber under vacuum, fractured using a pre-cooled blunt rod and then coated with carbon as described above. When coating was completed, the frozen samples were transferred under vacuum to the cold stage (maintained at -165°C) of the Hitachi S570 SEM and then examined and/or analysed using a working distance of 18 mm. The products prepared in this way included: 1) two white chocolates with different particle reduction (refining) parameters in manufacture and containing 20% milk solids but no cocoa solids, 2) plain chocolates containing 18-20% fat-free cocoa solids but...
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Fig. Modified EMscope specimen holder containing a slotted carbon insert into which the specimen (white chocolate) is secured with carbon cement.

nominally no milk and 3) several examples of crumb and dry mix milk chocolates containing both 5-6% cocoa solids and 20% milk solids. From each type of chocolate, many X-ray maps were obtained from which the results given below were derived.

**X-ray microanalysis**

All X-ray microanalysis was done using a Link Analytical AN 10/85 spectrometer with a standard windowed detector fitted to the specimen chamber of the SEM. When areas of the specimen had been selected for elemental analysis, the lowest accelerating voltage was used (15kV) that would give good X-ray yield without loss of spatial resolution caused by excessive penetration of the electron beam into the specimen. X-ray energy spectra were obtained using a 100 s count time with X-ray count rates of 1700-1900 counts/s. Conditions for X-ray emission were standardised by keeping constant the microscope variables of accelerating voltage, spot size, aperture and filament current.

The spatial distribution of a number of elements (including calcium, phosphorus, chlorine and potassium) was determined using a semi-quantitative, dead time corrected, digital X-ray mapping procedure which was chosen because it overcame the problem of intensity distortion experienced with simple mapping techniques. This technique involved the selection of element specific energy regions (windows) from the X-ray spectrum together with background windows which were used to subtract noise and eliminate topography-induced changes in signal level from the window integrals. X-ray maps with resolutions of 128 x 128 or 512 x 512 pixels and dwell times varying from 300 ms to 600 ms were obtained from a number of chocolate samples. For frozen specimens, it was found to be imperative to use dwell times of at least 300 ms to obtain maps of sufficient quality for later use.

In the course of this mapping procedure, partial overlap of the potassium Kα with the adjacent calcium Kα peak of the spectrum was largely avoided by using only those X-rays near the energy peaks of potassium Kα and calcium Kα for mapping. The separation of potassium from calcium distributions in this way was so important for the conclusions of this study that the results obtained from a given area by integrating windows were then frequently checked. Without moving the specimen, a quantitative elemental mapping programme (Remote Quantitation, Link Analytical) was used at low screen resolutions e.g. 64 x 64, but with dwell times of at least 1.5 s and incorporating the ZAF - PB quantitative software with the use of proper standards from Microanalysis Consultants. The advantages of this new software include: a) the ability to map quite separately, elements whose energy peaks partially or completely overlap, b) automatic elimination of background by digital filtering throughout the energy range and c) the application of rigorous statistical significance tests to maps of low concentration to reveal true low concentration areas; the latter can be a thorny problem in digital X-ray mapping techniques. The statistical significance of the calcium and potassium distributions (or any other elements of interest) obtained in this way was checked by processing the maps and adjusting to zero those pixel intensities which were less than 1.2 or 3 standard deviations in magnitude. Thus, if the intensity of a given
pixel in an image is, for example, more than 3 times the value of its standard deviation, it is almost certain (99.9%) that there is some of the element at that location. The development of this procedure has been discussed at length by Statham (1988a, 1988b).

Different levels of the elements in the finished X-ray maps were represented by a range of pixel display colours. Results were recorded on Kodachrome 200 colour film using a Celtic camera device in parallel with the spectrometer display screen.

Quantitative measurements of particles imaged in X-ray maps included mean diameter, area, coordinates of centre of gravity, Feret diameters (maximum and minimum) together with orientation and shape analysis. When required, these parameters were computed using binary images of the X-ray maps in the Link Analytical Digiscan feature analysis programme. Particle size and area distributions were plotted using these data. Where required, cumulative distributions from different samples were compared using the Kolmogorov-Smirnov Two Sample Test (Siegel, 1956) because of its high power-efficiency.

Results

The X-ray energy spectra, obtained from areas of spray dried milk powder and from dried cocoa solids, consistently showed that both contained significant amounts of phosphorus and potassium but that only the particles of dried milk produced an additional calcium peak (Figs 3 & 4). To eliminate the possibility that small amounts of calcium in the cocoa solids were being missed in this type of area analysis, high resolution (512 x 512) digital X-ray maps of preparations of cocoa solids mounted on carbon stubs were produced and the distribution of potassium compared with that of calcium. The results showed that potassium was distributed continuously throughout the particles of cocoa solids but that its concentration, even within a single particle, could be highly variable (Fig. 5). Moreover, although X-ray maps of calcium distribution in the same fields demonstrated that some cocoa solids contained small focal concentrations of calcium (Fig. 6), subsequent conversion of the maps to binary images followed by area measurements demonstrated that the mean area occupied by calcium was only 0.9% (+S.D.) of the area occupied by potassium, which was 0.5, number of fields = 15). It is important to note that calcium frequently occurred in areas where potassium levels were lowest.

In the case of milk powder, digital X-ray maps demonstrated that particles contained similar levels of uniformly distributed potassium, calcium, phosphorus and chlorine. These results were consistent with the relative levels of the same elements given by Ling, Kon & Porter (1961) and Kirchgessner, Freesecke & Koch (1967) for liquid bovine milk (K, 0.15; Ca, 0.12; Cl, 0.11; P 0.10 g/100g). However, an apparent slight reduction in concentration of all of these elements was observed at the periphery of the spherical powder particles, which could be explained as lower X-ray collection from curved edges.
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surfaces which sloped away from the detector. It appeared from these results that it would be possible using cryo-SEM in conjunction with X-ray microanalysis to determine the spatial distribution of cocoa solids in intact plain chocolate by comparing maps of potassium distribution with those of calcium. As a result of the observations described above, it could be predicted that, since milk powder is often added only to the extent of 1-2% as an anti-bloom agent or as a minor component of added butterfat, areas containing calcium would be minimal in this type of chocolate. However, the X-ray mapping results, given in Figs 7 & 8, showed large potassium rich cocoa particles distributed throughout the chocolate and small areas which, because they were rich in calcium but contained very low levels of potassium close to the limit of detection, were also identified as cocoa solids. Moreover, small and occasional particles were also found which contained high levels both of potassium and calcium and which were therefore identified as milk protein. These observations also provided the opportunity to establish that milk protein can be positively identified in digital X-ray maps of chocolate by the presence not only of calcium and potassium but also of phosphorus and chlorine at similar levels in the same areas. However, cocoa solids were always rich in potassium but contained variable levels of phosphorus together with the traces of calcium referred to above. These differences in elemental content were quite sufficient to allow both populations of particles to be identified and distinguished, as will be demonstrated below.

From a comparison of the area analyses of skinned milk powder (Fig. 4) with frozen and fractured white chocolate (Fig. 9), it would be expected that digital X-ray maps of the latter, which contains no cocoa powder, would possess areas of milk solids equally rich both in calcium and potassium. Thus, the X-ray maps of white chocolate seen in Figs 10 & 11 show large particles which could be positively identified as milk protein from their elemental composition. Similar maps were obtained for the distributions of phosphorus and chlorine. The particulate nature of the milk also clearly demonstrated that the chocolate was of the dry mix type in which milk powder rather than liquid or condensed milk is added to the rest of the ingredients. In another commercially produced white chocolate (B) containing the same amount of milk solids, the distributions of calcium and potassium seen in X-ray maps (Figs 12 & 13) showed that it too was of the dry mix type but that the particles appeared to be generally smaller than those seen in Figs 10 and 11. This was confirmed by comparing the distributions of the various particle parameters using the data obtained by processing 10 X-ray images within the Digiscan feature analysis software. As an example, particle area distributions in these two chocolates are shown in Figs 14 and 15. Referring to the cumulative distributions (Fig.16) and by application of the Kolmogorov-Smirnov Two Sample Test (maximum deviation in cumulative distributions = 0.097; critical value from tables = 0.082) they can be seen to be significantly different (p < 2.5% but 1%) with one of the samples (B) containing a higher proportion of smaller particles than the other (A).

Whilst the ability to discriminate between the populations of milk powder particles in two different white chocolates in this way demonstrates one application of the technique, its use with milk chocolates poses a problem similar to that seen above with plain chocolate, i.e., one of distinguishing between the different element profiles of cocoa solids and particles of milk solids in a single matrix. Given that most plain chocolates contain approximately 20% non-fat cocoa solids whereas milk chocolate contains around 5% (Cook, 1982; Jackson, 1988), it is to be expected that the cocoa solids in the latter would be proportionately reduced in number in X-ray maps and that the milk solids would be increased.

An example of the X-ray energy spectrum derived from an area analysis of a frozen and fractured dry mix milk chocolate is shown in Fig. 17. The secondary electron image of such a chocolate warmed to 28°C and then frozen and fractured can be seen in Fig. 18; particles which can be tentatively identified as sugar crystals and milk powder grains are clearly visible. The results obtained from digital X-ray mapping showed that, as with the dry mix white chocolates, milk protein was present predominantly as numerous dispersed and separate particles whereas the cocoa solids with their very low levels of calcium and relatively high levels of potassium were sparse and generally somewhat smaller (Figs 19 & 20).
Fig. 14 Percentage distribution of the areas of milk particles derived from 10 X-ray maps of chocolate A (n = 504).

Fig. 15 Percentage distribution of the areas of milk particles derived from 10 X-ray maps of chocolate B (n = 605).

Figs 5 - 6. Digital X-ray maps (512 x 512) of cocoa solids isolated from cocoa mass. Dwell time 600 ms; 15kV.
Fig. 5 Potassium distribution. The central particle containing high levels of potassium may be derived from the seed embryo.
Fig. 6 Calcium distribution.
Figs 7 - 8. Digital X-ray maps (128 x 128) of frozen and fractured plain chocolate. Dwell time 300 ms; 15kV.
Fig. 7 Potassium distribution.
Fig. 8 Calcium distribution. The particle at lower left also possesses high levels of potassium (see Fig. 7) and is therefore identified as milk powder. The other area rich in calcium has no obvious counterpart in Fig. 7 and is a particle of cocoa solid.
Figs 10 - 11. Digital X-ray maps (128 x 128) of frozen and fractured white chocolate A showing the distribution of milk proteins. Dwell time 300 ms; 15kV.
Fig. 10 Potassium distribution.
Fig. 11 Calcium distribution - same area as that seen in Fig.10. Note the coincidence in position of potassium and calcium rich areas.
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INCREASING CONC. →
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Figs 12 - 13. Digital X-ray maps (128 x 128) of frozen and fractured white chocolate B showing the distribution of milk proteins. Dwell time 300 ms; 15 kV.

Fig. 12 Potassium distribution in white chocolate B.

Fig. 13 Calcium distribution in white chocolate B. Same area as in Fig. 12.

Figs 19 - 24. Digital X-ray maps (15 kV) of frozen and fractured dry mix milk chocolates derived from the same field of view using:

Figs 19-20 window integrals - dwell time 300 ms;

Figs 21-22 remote quantitation (ZAF PB) - dwell time 2000 ms;

Figs 23-24 quantitative mapping (ZAF PB) standard deviation corrected for statistical significance - dwell time 2000 ms.

Fig. 19 Potassium distribution in dry mix chocolate. Large particles contain high levels of potassium (128 x 128).

Fig. 20 Calcium distribution in dry mix chocolate. Large particles containing high levels of calcium also contain potassium (compare with Fig. 19) and are identified as milk protein. (128 x 128).

Fig. 21 Quantitative map showing the distribution and apparent concentrations of calcium. Notice several large particles identical to those seen in Figs 19 and 20 and containing high levels of calcium. They are particles of milk powder. (64 x 64).

Fig. 22 Quantitative map showing the distribution and apparent concentrations of potassium. By reference to Fig. 21, notice that some of the particles containing high levels of potassium do not contain calcium and can be therefore be identified as cocoa solids. (64 x 64).

Fig. 23 Quantitative calcium distribution map seen in Fig. 21 processed for statistical significance. Pixels are set to zero if their intensity is less than 2 standard deviations. (64 x 64).

Fig. 24 Quantitative potassium distribution map seen in Fig. 22 processed in the same way as Fig. 23 for statistical significance. The differences between the distributions in Figs 21 and 22 are real because they persist in Figs 23 and 24. (64 x 64).

The application of Remote Quantitation (quantitative mapping) to dry mix milk chocolate was used to: a) verify the reliability of using window integrals to map the separate distributions of calcium and potassium which have partially overlapping K peaks, i.e., that calcium distributions were not distorted by contributions from potassium Kα X-rays and b) test the statistical significance of areas believed to be cocoa solids but containing low levels of potassium. In quantitative X-ray maps (Figs 21 - 24), the distributions and levels of calcium (Figs 21, 23) were found to be similar to those seen in digital maps of the same area obtained using window integrals and background subtraction (Fig. 20), demonstrating that, under the conditions used in the present study for the production of window integral maps, the contribution of potassium Kα X-rays to calcium distribution was insignificant.

Fig. 17 Part of the X-ray energy spectrum from a frozen and fractured dry mix milk chocolate. Area analysis; 15 kV.

Fig. 18 Scanning electron micrograph of frozen and fractured dry mix milk chocolate showing putative fractured sugar crystals (S) and particles of milk protein (P). 16 kV.

Although cocoa solids in milk chocolate could usually be identified using the criteria established above with isolated cocoa solids and plain chocolate, it was difficult to be categorical when confronted with particles containing very low levels of potassium and/or which were only 1 or 2 pixels in area (Fig. 19). However, a quantitative map of the same area showing the distribution of potassium (Fig. 22)
The ability to image chemically defined populations of particles in this way permits the quantitative evaluation of parameters such as area, aspect ratio (ratio of length to width), orientation, shape and Feret diameters using the software described above. Rostagno (1960) has emphasized the importance of particle size to the organoleptic properties of chocolate and has reviewed some of the methods available for determining the size distributions of solids isolated from chocolate products by extraction; but they lack spatial resolution and permit neither discrimination between populations of various solids nor the measurement of individual particle parameters in situ. It is worth noting that isolation of the particulate components of chocolate by fat extraction can lead to aggregation and difficulties in redispersion but it is widely used in industry to prepare solids for size distribution determinations and has been employed by some workers to study the effects of processing on size and shape of sugar and cocoa solids (Hoskin & Dimick, 1980).

The derivation of such information for cocoa and milk solids and sugar particles is necessary if a critical analysis of the rheological and mechanical properties of intact chocolate is to be made. Thus, it is already known that the particle size distributions greatly affect the rheological properties of chocolate (Prentice, 1984a; Chevalley, 1984). In particular, the Casson yield value is related inversely to particle size, supposedly as a result of increased frictional contact or stronger bonds between the smaller particles, but the complex effects of particle size on Casson viscosity are, according to Prentice (1984a), more difficult to explain in terms of the structural information currently available. Likewise, the mechanical properties of food composites such as chocolate, i.e. foods consisting of a continuous matrix containing dispersed particles, are influenced not only by the material properties of the continuous and discontinuous components and the volume fraction of the particles, but also by particle shape, size, orientation and their state of adhesion with the matrix (Chow, 1980; Richardson et al., 1981; Ross-Murphy & Todd, 1983; Langley & Green, 1989).

Using transmission electron microscopy, Heathcock (1993) has demonstrated the role of protein (in the form of casein micelles) in crumb based chocolates is very evenly distributed, but that in dry mix products containing full cream or skimmed milk powder, it is particulate. The results obtained in the present study using dry mix and crumb chocolates are in agreement with these observations and extend them by demonstrating that it is possible, using digital X-ray mapping of bulk samples, to distinguish in situ between dry mix products having different milk protein particle size distributions. It should be emphasized that the major differences between these two approaches are that cryo-SEM in conjunction with X-ray microanalysis is relatively rapid, identifies milk powder and cocoa solids on the basis of elemental composition rather than on morphology and permits morphometric analysis of large (relatively) samples. However, contrary to expectation, it was not found necessary to melt chocolate by warming to 40°C before freezing; in chocolate prepared only to 28°C, specimen preparation was easier than at ambient temperature and although a layer of cocoa butter may have
overlain those particles exposed by fracturing, it appeared to have been of insufficient thickness to affect significantly either secondary electron imaging or X-ray microanalysis.

Acknowledgement

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References


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Discussion with Reviewers

G.M. Roomans: Preparation of cocoa mass for X-ray microanalysis includes a defatting step with chloroform and methanol. Have you checked the effect of this treatment on the elemental composition?

Author: Yes. I have obtained identical results using cocoa powder instead of solvent extracted cocoa solids.
B.E. Brooker

G.M. Roomans: Some of the spectra suggest that the intercept of the spectra with the x-axis is considerably below 15 keV (theoretically the intercept should be at the value of the accelerating voltage used). This would indicate severe charging of the specimen. Is this correct?

Author: No. The spectra originally sent to the referees were printed in a compressed form to give maximum detail of the energy peaks but in the process they have given the impression that you have referred to. The spectra to be published have been printed to show the X-ray energy range (intercept with the x-axis) more clearly. The intercept with the x-axis is very close to 15 keV. None of the specimens were charging during the acquisition of X-ray data because they were well coated with carbon. This is a sine qua non for the use of this technique with such non-conducting specimens.

D. Manning: Is it possible to take a SEM micrograph of a specific area on a sample and then follow with X-ray spectroscopy to clearly identify those shapes observed?

Author: Yes. So called real time imaging, in which elemental maps are acquired as you watch, allow areas rich in certain elements to be related to corresponding structures in areas in the secondary electron image. This can be done accurately by electronically superimposing the two images. Most modern X-ray systems have the ability to perform this routine procedure.

Another approach is to use the spectrometer to capture and store a secondary electron image of the field to be examined and to then move the electron beam onto the individual particles of interest and collect the X-ray spectra. Particles of cocoa solids and milk protein can be distinguished on the basis of differing elemental compositions as described in this paper.

However, it is worth noting that although the procedure can be used to identify individual particles seen in micrographs of chocolate, the primary intention of this study was to demonstrate that the shape, size and spatial distribution of milk and cocoa solids can best be determined directly (and without recourse to SEM) using X-ray mapping techniques.

D. Manning: How does particle size reduction influence the results of the X-ray spectroscopy? Would superfine particles produced during milling influence the X-ray spectroscopy results? How would the numerous ways used to reduce particle size influence these results (i.e. roll refiners, ball mills, etc.)?

Author: X-ray mapping is a sensitive way of determining the effect of particle size reduction methods on size distributions in intact chocolate. This study has shown that extending the refining process has a significant influence on size distribution by increasing the number of small particles. Milk and cocoa solids which are only 1 - 2 μm in diameter can be detected in this way and included in the distributions provided the magnification used for mapping is sufficiently high. However, the technique is not suitable for particles smaller than this and therefore those that are 'superfine' will not be included.

One of the reasons for developing this technique was to answer the sort of questions you have posed here about refining methods. A study of this kind is currently underway.

J. Heathcock: The cocoa solids in chocolate are small fragments of the original whole cocoa bean tissue and are much likely to differ in composition. Have you been able to make any chemical distinction between, for example, cell wall fragments and starch granules? Have you any ideas as to the origin of the calcium in the cocoa solids?

Author: Individual particles of cocoa solids do differ considerably in their elemental composition. This variation with respect to potassium levels can be seen in Fig. 5, but levels of phosphorus and magnesium are also markedly variable between particles. Work in this laboratory suggests that these observations reflect the chemical differences between anatomically distinct tissues such as seed embryo and cotyledon. No attempt has been made to study chemical differences at the level of cellular organelles and inclusions with SEM; this aspect is more likely to yield meaningful results if studied at higher spatial resolution and for this reason it would be more appropriate to use transmission electron microscopy in conjunction with X-ray analysis.

It appears that most of the calcium in the cocoa solids is derived from focal concentrations seen in peripheral cells of the cotyledon.

J. Heathcock: Have you any ideas or explanation for why a dwell time of > 300 ms was necessary in the analysis?

Author: It is important to realise that the emission of X-rays from excited atoms is a random process. If the dwell time for X-ray mapping is too short, too few X-rays may be generated from the specimen to register a value above background. Under these circumstances, elements may not be detected even if they are present at quite high levels.

A long dwell time allows relatively large numbers of X-rays to be collected from any given part of the specimen, thereby increasing the accuracy with which element concentration is calculated by the software.

For this reason, it is widely advocated that the dwell time should be as long as is practically possible but not shorter than 150 ms. Since it was important in the present study to get the highest possible precision with particle outlines for diameter measurement, 300 ms was chosen for routine mapping.

J. Heathcock: The preparation of cocoa mass involved the crushing and the spreading of the particles prior to analysis. Was any similar procedure used for the milk powder reference samples in order to obtain analytical data on the internal and the external surfaces?

Author: Yes, as described in Materials and Methods of this paper. Fractured particles of skimmed milk powder produced X-ray maps in which there was uniform and high levels of calcium, phosphorus, potassium and chlorine. The
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appearance of intact particles is described in Results. Colour X-ray maps were not included because of the shortage of space.

J. Heathcock: Have you any comments from your work on the likely distribution of the remaining components, fat and sugar, within chocolate? 

Author: Using the technique described in this paper it is possible to identify particles of sugar with some certainty once the milk and cocoa solids have been located. This is best done by superimposing the X-ray map on the SEM image as described above.

J. Heathcock: The paper only covers skimmed milk powders rather than other sources of milk powder. Have you studied chocolates containing other powders or mixtures of powders, in particular, full cream milk powder or whey?

Author: Yes. We have examined chocolates containing full cream milk powder, or mixtures of skimmed milk powder and crumb, or whey powder. The results obtained are generally rather similar but some differences do occur which have not been included in this paper but which will be published as part of another study.

J. Heathcock: Can you please explain the term area analysis?

Author: This is a spectral analysis in which X-rays are collected from the full area of the specimen displayed on the SEM. This is different from a point analysis in which the electron beam is placed on one point of the specimen for X-ray generation.

J. Heathcock: Low temperature fracturing through a chocolate is likely to result in fracture planes that run over and under, as well as through the middle of particles. Does your particle size work take account of this in the calculations?

D. Lewis: With any fracturing process the size of particle 'seen' depends on the fracture plane through that particle and correction procedures are possible to compensate for this. A realistic assessment requires counting a lot of particles and preparing digital X-ray maps at high magnifications will make this a very time consuming exercise. How do you propose to make this compensation in your critical analysis of intact chocolate?

Author: The acquisition of particle information at the appropriate magnifications can be a consuming exercise if it is not approached the right way. In our laboratory, data acquisition is now done during the night and at week ends when the instruments are not otherwise in use. This requires installing an automatic liquid nitrogen top-up device to replenish the nitrogen dewar cooling the microscope stage so that specimens can be kept cold for at least 48h. In addition, it is necessary to use one of the commercially available software packages to control the movement of the specimen, via stepping motors on the microscope drives, which will move the specimen to many new areas for mapping once the previous one has finished and has been stored on disc. Performed in this way, a great deal of data can be obtained without much effort.

It is true that the fracture plane passes over, as well as through, the particles to be analysed. As Dr Lewis has commented, it is possible to compensate for this using correction procedures but the size distributions given in this paper were not processed in this way to give absolute values. The intention was simply to show that it is possible to demonstrate the relative shift in particle distributions caused by a change in one of the chocolate processing steps. Since the error in the uncorrected data is the same for both samples, this comparison is legitimate. As far as correction procedures are concerned, it should be remembered that, because of penetration effects by the electron beam, each particle in an X-ray map is a projection derived from surface and some sub-surface information. Each map can therefore be treated as a section through the chocolate and appropriate correction treatments applied. We have found a modification of the scheme proposed by Bach (Biometrische Zeitschrift, 18, 407-412, 1976) to be useful in this context.

Of greater concern to us is the fact that fracture planes represent lines of greatest mechanical weakness in the specimen and cannot therefore be assumed to reveal structure which is representative of the specimen as a whole. For this reason we are now milling the frozen specimens to produce a flat surface rather than fracturing. Provided the cutting tool is sufficiently sharp (e.g. diamond) particles are cut cleanly with no smearing and X-ray mapping produces a 'section' of the specimen. Particle diameters can then be processed using standard morphometric techniques to give an accurate size distribution. This approach allows any number of planes within the specimen to be examined and for the whole process of specimen preparation to be closely under the control of the operator.

D. Lewis: Modified milk products are available and could be used to make chocolate. Don't you think that this technique needs to be coupled to other microscopical approaches to be successful?

Author: Obviously, studying the properties and interactions of particles in chocolate should not be constrained by the use of only one microscopical technique. However, the technique described in this paper is not directly coupled to other microscopical methods yet provides separate quantitative information for cocoa solids and milk protein in bulk samples. In this particular respect, I believe that it surpasses other microscopical techniques.

As mentioned above, for chocolate made from full cream milk powder or whey powder, the results obtained by X-ray spectrometry are good with clear discrimination between milk and cocoa solids. However, if, by modified milk products, you are referring to materials, such as demineralized whey, which contain little or none of the elements used in this study to locate milk, then clearly, the technique could be used to characterize only the cocoa solids.
MICROSTRUCTURE AND FIRMNESS OF PROCESSED CHEESE MANUFACTURED FROM CHEDDAR CHEESE AND SKIM MILK POWDER CHEESE BASE

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Abstract

Processed cheese (10 different types) was made from Cheddar cheese and a cheese base produced from reconstituted skim milk powder by blending and melting with commercial emulsifying salts at 90°C. In one experiment, the cheese base was subjected to accelerated cheese ripening by added enzyme. The finished products had 50.1-53.5% total solids, 18.2-19.3% protein, 47.4-49.7% fat in dry matter, and 2.7-3.0% salt in water; pH was 5.3-5.4 after three months of storage at 10°C and 30°C.

The experimental cheeses were markedly firmer than the control cheeses. All processed cheeses exhibited a similar pattern of firmness whereby the samples stored at 10°C were firmer than the fresh cheeses and the cheeses stored at 30°C were firmest. Only blends containing a large proportion of a cheese base treated with added enzyme were crumbly and were not satisfactory.

Electron microscopy revealed differences in the structures of the raw materials and the processed cheeses. The cheese base, to which a protease was added, had an open structure compared to a compact structure of the untreated cheese base. The microstructures of all the finished processed cheeses stored at 10°C were similar to each other. Storage of these cheeses for 3 months at 30°C resulted in the development of irregularly shaped fat particles, but differences in their dimensions were statistically not significant.

Introduction

Different types of processed cheese have been manufactured successfully on a large scale in Europe and the United States of America since the beginning of this century. Meyer (1973) has provided an excellent historical background of the development of processed cheese, and recently various aspects of the manufacture of processed cheese have been reviewed by many authors (Kosikowski, 1977; Thomas, 1977; Carić et al., 1985; Carić and Kaláb, 1987). In brief, the product is primarily made by blending natural cheeses (young, mature, or different types) in the presence of water, colouring matter, emulsifying salts and other dairy ingredients, and then heating and agitating to produce a homogenous mixture.

Such products have gained consumer acceptability over the years. They may be classified into three different types which are referred to as block variety, slices, and cheese spread. In certain countries, e.g., the United States of America and Canada, the reliance on natural Cheddar cheese for the production of processed cheese (i.e., block and slices) is apparent. Prolonged storage of Cheddar cheese is required to achieve the maturation process and this could be a disadvantage to the processed cheese manufacturer because of high costs.

Recently, Ernstrom et al. (1980) have achieved the production of cheese base from whole milk which could be used in a processed blend. For example, a blend of 80% cheese base and 20% matured Cheddar cheese was suitable for the manufacture of processed cheese. A similar method for the production of cheese base in Europe and the United States was reported by Madsen and Bjerre (1981) and Rubin and Bjerre (1983a, 1983b) who recommended the use of similar proportions of cheese base and Cheddar cheese as those reported by Ernstrom et al. (1980) or lower, i.e., 50:50 ratio. Other patent applications for the production of cheese have been reported by Jameson and Sutherland (1986) and Moran et al. (1989).
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The method of cheese base production could be briefly described as follows: (a) standardisation of the fat content in milk, (b) ultrafiltration (UF) of the milk to concentrate the protein, (c) diafiltration of the retentate to reduce the lactose content, (d) acidification of the concentrate (i.e., addition of starter culture) to lower the level of calcium content in the casein micelles, and (e) vacuum evaporation to remove the excess moisture. The composition of the resulting product is similar to Cheddar cheese.

In Egypt, large volumes of processed cheese are imported every year. In 1984, the total cheese imports were 42,000 tonnes, of which 10,500 tonnes was processed cheese (IDF, 1986). In economic terms, the imported cheese was valued at 82 million US $s. In order to reduce cheese imports, a collaborative programme of work was established two years ago between the West of Scotland College and University of Zagazig in Egypt for the development of cheese base production from reconstituted skim milk powder for local manufacture of processed cheese.

The purpose of this research work was to examine the effect of 2 cheese bases (one without and the other with added proteolytic enzyme to induce proteolysis) produced from reconstituted skim milk powder on the microstructure and firmness of block type processed cheese.

Materials and Methods

Materials
While Cheddar cheese (young, 5 months old, and mature, 11 months old) and medium heat skim milk powder (whey protein index - 4.5 mg N/g powder) were obtained from Scottish Pride Quality Dairy Foods Ltd., Galloway Creamery, Stranraer, Scotland.

Anhydrous milk fat (AMF) (Aberdeen and District Milk Marketing Board, Buckburn, Aberdeen, Scotland) was added to all processed cheese blends except the control (Table 1) in order to maintain a constant fat content in the cheese; this ingredient was added to the melting cooker with the other additives.

Pure vacuum-dried sodium chloride (ICI Chlor-Chemicals, Cheshire, UK) was used in the manufacture of the cheese base and in the processed cheese formulations.

Production of cheese base
Cheese base was produced as reported by Younis (1989) and Tamime et al. (1990). In brief, this process may be described as follows:

Skim milk powder was reconstituted at 20% total solids using water at 50°C (total weight 1200 kg) followed by ultrafiltration (UF) (APV Baker Ltd., Preston, UK) to a 2-fold concentration at 50°C by removing 600 L of permeate. Water, equal to the volume of permeate removed, was added to the retentate and diafiltration of the milk was carried out to a 2-fold concentration at 50°C by removing 600 L of permeate. The retentate was pasteurised at 72°C for 15 s using a plate heat exchanger (APV Baker Ltd., Crawley, UK), cooled in it to <10°C, and stored overnight in a refrigerator.

On the following day, the retentate was warmed to 32°C and ripened with a multiple strain mesophilic cheese starter culture (Streptococcus lactis sub-sp. lactis and St. lactis sub-sp. cremoris code MAO11C from Eurozyme Ltd., London, UK), which was added at 2 g/10 kg retentate until the pH reached 5.8 ± 0.1. The fermented retentate was coagulated in an ALCURD machine MK III (Alfa-Laval Eng., Ltd., Middlesex, UK) for 10-12 min using standard calf rennet (Chr. Hansen's Lab. Ltd., Reading, UK) added at 2.5 mL/10 kg retentate. The curd was delivered to an open top cheese vat, mixed gently with stainless steel forks until pH of the whey dropped to 5.6 ± 0.1, and the curd and whey were heated indirectly to 39°C in 15 min.

Cheese Base I. After draining the whey (i.e., a very small volume was removed as compared with the conventional cheesemaking process), the curd was salted at 2.5% (w/w), mixed for 15 min, hooped into a 20 kg rectangular Cheddar cheese mould, pre-pressed for 1 h at an air line pressure of 0.27 MPa, (the pressure was acting in a 20 cm diameter cylinder) and pressed overnight at 0.5 MPa. On the following day, the pressed curd was divided into 4.5 - 5 kg blocks, placed in BD-1 bags (W.R. Grace Ltd., London, UK), vacuumed, heat-sealed, and shrunk in hot water at

| Table 1. Formulations (%) of major ingredients in processed cheese blends |
|-----------------------------|-----------------|-----------------|------------------|-----------------|-----------------|
| Processed Cheese            | Cheddar Cheese  | Cheese Base     | AMF              | Added Water     |
| Control                     | 19.7            | 59.2            | -                | 26.5            | 18.1            |
| Exptl.                      | -               | 42.2            | 14.1             | 20.5            | 14.3            |
| Blend A                     | 20.5            | 41.8            | -                | 19.9            | 14.8            |
| B                           | 20.0            | 40.0            | 13.6             | 6.4             | 17.1            |
| C                           | -               | 59.7            | 13.6             | 6.3             | 17.5            |
| D                           | 13.2            | 59.4            | 4.5              | 2.1             | 17.9            |
| A                           | 20.5            | -               | 41.6             | 19.6            | 15.3            |
| B                           | 20.0            | 39.9            | 13.6             | 6.4             | 17.2            |
| C                           | -               | 59.7            | 13.6             | 6.3             | 17.5            |
| D                           | 13.2            | 59.3            | 4.5              | 2.1             | 18.0            |
* M: mature; Y: young; Cheese Base I: with no added enzyme; Cheese Base II: with added Savorase-A; AMF: anhydrous milk fat. Sodium chloride, Nisin and emulsifying salts were added to each blend at 0.44%, 0.01%, and 2.50%, respectively.
Processed Cheese Made from Skim Milk Powder Cheese Base

85°C. The cheese base was stored at 10°C until required for processing.

Cheese Base II. The drained curd was salted with 1.13% (w/w) of salt and mixed with a dried enzyme preparation from Stt. lactis sub-sp. lactis (Savorase-A, marketed by Imperial Biotechnology Ltd., London, UK) at a rate of 1.65% (w/w) (the enzyme preparation was a cell-free extract and contained 50% w/w sodium chloride), and mixed for 15 min. The salted curd was handled afterwards as described above for cheese base I. According to the supplier, the Savorase-A activity was expressed as 5.3 flavour amino peptidase (FLAP) units/g.

Production of Processed Cheese

Ten different formulations of processed cheese (Table 1) were manufactured as follows: The cheese ingredients were cut into small cubes by hand and blended in a Stephan cooker, type UMM SK40E (A. Stephan u. Söhne, GmbH & Co., Hamelin, West Germany) for 1 min at 750 rpm. Different amounts of AMF, sodium chloride, and water were added to each blend including 1.4 g Nisin (Alpin and Barrett Ltd., Wiltshire, UK) which was used as a preservative, and the mixture was blended for 1 min at 750 rpm. Joha SE, C, and T (Fibrisol Services Ltd., London, UK) commercial emulsifying salts were used at a ratio of 1.25 : 1.0 : 0.75 at the total level of 3% (w/w). They were blended with the other ingredients for 1 min at 1500 rpm. Direct steam injection was used to heat and melt the cheese ingredients and blending continued at 1500 rpm until the temperature reached 85-90°C. Vacuum was applied for 1 min to remove air bubbles and stirring was continued for 2 min. The melted cheese was hot-filled in cartons lined with commercial pouches (Pukkafilm, DRG Flexible Packaging Ltd., Bristol, UK), sealed, and cooled in a refrigerator for 3 days at 5°C; the cheese samples were then stored for 6 months at 10°C and 30°C. The mass of each batch was 13.6 kg.

The pouches (185 x 380 mm) had the moisture vapour transmission rate of 0.8 - 1.0 g/m²/24 h at 25°C and 75% relative humidity and the oxygen permeability rate of 20 cm²/m²/24 h at 20°C and 0% relative humidity.

Chemical Analysis

Total solids, fat, salt, phosphorus (total), and pH were determined according to British Standards Institution methods (BSI, 1969 and 1976). Calcium and soluble nitrogen were determined by the methods described by Pearce (1977) and Kosikowski (1977, p. 572), respectively.

Casein hydrolysis was determined using polycrylamide gel electrophoresis according to the method reported by Ridha et al. (1984).

Firmness Analysis

A Stevens LFRA Texture Analyser (C. Stevens & Son Ltd., Hertfordshire, UK) equipped with a type TA26/TFE-105-524Y probe was used to assess firmness of the processed cheese. The U-shaped probe, with a wire connection, 0.33 mm in diameter, penetrated the samples into a depth of 15 mm at a rate of 0.5 mm/s. The chart recorder was operated at 500 mV and 30 mm/min chart speed. The results were expressed in newtons (N).

The firmness measurements were carried out on processed cheese samples that had been tempered at 70°C for 3 days and cut into blocks of 50 x 20 x 20 mm.

Microscopic Analysis

Processed cheese was sampled by cutting a slice about 10 mm thick which was then sectioned into columns 10 mm wide and 25 mm long. Sample columns were fixed in a 2.8% aqueous glutaraldehyde solution and mailed to Ottawa for electron microscopy (Allan-Wojtas, 1984). After arrival, the samples were cut into prisms, 1 x 1 x 15 mm, for scanning electron microscopy (SEM) and into cubes, ~0.6 mm on a side, for transmission electron microscopy (TEM), and placed into a fresh glutaraldehyde solution for 2 h.

For SEM, the cheese prisms, fixed with glutaraldehyde, were washed with water and subsequently dehydrated in a graded (20, 40, 60, 80, 96, and 100%) ethanol series. The prisms impregnated with absolute ethanol were defatted by extraction using 3 changes of chloroform, returned into ethanol, frozen in Freon 12 cooled to its freezing point with liquid nitrogen, and placed in liquid nitrogen, where they were fractured. The fragments were critical point-dried from carbon dioxide, mounted on aluminium SEM stubs, sputter-coated with gold, and examined at 20 kV in an ISI DS-130 scanning electron microscope equipped with an external oscilloscope (Bond and Kaláb, 1988). Micrographs were taken on 35 mm film.

For TEM, the 0.6 mm cubes were washed with a 0.05 M veronal-acetate buffer (pH 6.75) and were postfixed for 2 h with a 2% osmium tetroxide solution in the same veronal-acetate buffer. Then, the cubes were embedded in Spurr's low-viscosity embedding medium (J. B. EM Service, Inc., Pointe Claire, Dorval, Quebec), and sectioned. The sections (approx 90 nm thick) were stained with uranyl acetate and lead citrate solutions (Reynolds, 1963) and examined in a Philips EM-300 transmission electron microscope operated at 60 kV. Micrographs were taken on 35 mm film.

Digital Image Analysis.

TEM micrographs of 9 μm x 10 μm areas, taken at a 20,000x magnification, were evaluated using a Kontron IBAS image analyser (Carl Zeiss Canada, Don Mills, Ontario, Canada) for the distribution of fat globule section diameters; in the case of irregularly shaped fat particles, their section areas were measured and the results expressed as the diameters of circles with equivalent areas.
Results and Discussion

Chemical Composition

Ten different blends of processed cheese under study were made from young and mature Cheddar cheese and cheese base I or II, or a combination of both; the formulations are presented in Table 1. In blends A and B, the young Cheddar cheese was replaced by cheese base I at different proportions, and in blends C and D, the mature Cheddar cheese was replaced by the same type of cheese base. A similar approach was used for blends A1 to D1 where cheese base II was used to replace young or mature Cheddar cheese.

Studies of processed cheese samples prepared according to the formulations shown in Table 1 were carried out in order to: (a) establish the feasibility of using a cheese base made from reconstituted skim milk powder in the manufacture of processed cheese, (b) compare the results obtained with the recommendations for various amounts of a whole-milk cheese base in processed cheese by other authors (Ernstrom et al., 1980; Madsen and Bjerre, 1981; Rubin and Bjerre, 1983a, 1983b), and (c) establish appropriate proportions of the developed cheese bases I and II so that they could be recommended for the production of block type processed cheese suitable for the Egyptian market.

Samples of each type of processed cheese were analysed when fresh and then again after 3 months of storage at 10°C and 30°C. Chemical composition of these cheeses is presented in Table 2 and data in greater detail have been published elsewhere (Younis, 1989). The total solids contents ranged between 50.1 and 53.5% and the fat contents in the dry matter ranged between 47.4 and 49.5% and, thus, met the Egyptian specifications for full-fat processed cheese, i.e., maximum 50% moisture and minimum 45% fat in dry matter (Egyptian Standards, 1970).

The level of sodium chloride (1.7%) and pH (5.4) were acceptable for block type processed cheese (Meyer, 1973; Kosikowski, 1977; Thomas, 1977). The calcium and phosphorus contents in Cheddar cheese and cheese bases I and II are shown in Table 3. It can be observed that the calcium content in the cheese bases was higher than in Cheddar cheese and the cheese bases contained approximately half the amount of phosphorus present in Cheddar cheese.

The firmness of the 10 various types of processed cheese (Figs. 1A and 1B) was affected by a number of factors such as:

(a) Type of cheese used: the firmness of the experimental processed cheese, which was made totally from cheese bases I and II, was 2.5 times higher than that of the control samples. (b) The addition of cheese base; blends

| Table 2. Chemical composition (%) of various processed cheese blends after storage for 3 months at 10°C and 30°C |
|-----------------|---------------|--------|----------|---|---|
| Proc. | Temp.* | Total Solids | FDM* | Protein | SM* | pH |
| Cheese | °C | | | | | |
| Control | 10.0 | 50.5 | 48.3 | 18.5 | 3.5 | 5.4 |
| | 30.0 | 50.8 | 49.7 | 18.4 | 3.4 | 5.4 |
| Exptl. | 10.0 | 51.9 | 47.4 | 18.9 | 2.9 | 5.3 |
| | 30.0 | 51.7 | 48.4 | 19.3 | 2.7 | 5.4 |
| Blend A | 10.0 | 50.8 | 48.3 | 18.6 | 2.9 | 5.3 |
| | 30.0 | 50.6 | 48.8 | 19.2 | 3.0 | 5.4 |
| B | 10.0 | 52.7 | 46.0 | 19.0 | 3.7 | 5.4 |
| | 30.0 | 52.8 | 48.9 | 19.3 | 3.6 | 5.4 |
| C | 10.0 | 52.9 | 48.0 | 19.0 | 3.4 | 5.4 |
| | 30.0 | 53.5 | 48.6 | 19.3 | 3.5 | 5.4 |
| D | 10.0 | 52.3 | 48.0 | 19.0 | 3.5 | 5.4 |
| | 30.0 | 52.4 | 49.2 | 19.0 | 3.5 | 5.4 |
| A1 | 10.0 | 50.1 | 49.5 | 19.1 | 2.8 | 5.3 |
| | 30.0 | 52.4 | 49.1 | 19.1 | 2.7 | 5.3 |
| B1 | 10.0 | 50.2 | 48.4 | 18.5 | 3.3 | 5.3 |
| | 30.0 | 50.4 | 48.8 | 18.3 | 3.1 | 5.3 |
| C1 | 10.0 | 50.9 | 49.2 | 18.6 | 3.3 | 5.3 |
| | 30.0 | 51.4 | 48.9 | 18.8 | 3.3 | 5.3 |
| D1 | 10.0 | 50.4 | 48.0 | 18.2 | 3.6 | 5.4 |
| | 30.0 | 50.7 | 49.3 | 18.3 | 3.5 | 5.4 |

* Temp.: Storage temperature; FDM: fat in dry matter; SM: salt in moisture; for details refer to Table 1. Results are the average of two duplicates from the same sample.

Table 3. Calcium and phosphorus contents (%) in cheese base and Cheddar cheese

<table>
<thead>
<tr>
<th>Product</th>
<th>Ca</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheese Base I*</td>
<td>1.02</td>
<td>0.45</td>
</tr>
<tr>
<td>Cheese Base II*</td>
<td>1.02</td>
<td>0.38</td>
</tr>
<tr>
<td>Cheddar cheese (Y)*</td>
<td>0.78</td>
<td>0.66</td>
</tr>
<tr>
<td>Cheddar cheese (M)*</td>
<td>0.82</td>
<td>0.64</td>
</tr>
</tbody>
</table>

* I, II, Y, M: see Table 1 for details. Ca: calcium; P: phosphorus.
Processed Cheese Made from Skim Milk Powder Cheese Base

Fig. 1. Firmness of processed cheeses made with varying amounts of cheese base I (A) or cheese base II (B) as compared with the control and experimental processed cheeses. CON: Control; EXP: Experimental; Fr. Fresh; 3M: Storage for 3 months; A, B, C, D, A1, B1, C1, and D1: Corresponding processed cheese blends; for details refer to Table I.

Containing the largest proportion of cheese base I or II were the firmest but a reduction in firmness was observed as the levels of cheese bases I or II were reduced in blends A-D and A1-D1, respectively. (c) Duration and temperature of storage: the firmness of all the processed cheeses markedly increased during storage and the cheeses became firmest by storage at 30°C. (d) Change in pH: all the cheeses exhibited a slight drop in pH from 5.5-5.6 when fresh to 5.3-5.4 after 3 months of storage. This change could be due to the presence of emulsifying salts and may have influenced some interactions that had increased the firmness of the cheese; it is known that as pH is decreased towards the isoelectric point of casein, the texture of processed cheese may become crumbly (Carić et al., 1985).

The firmness of the various blends of processed cheese, including the control and experimental samples, found after 3 months of storage at 10°C and 30°C, could be attributed to the interactions between the cheese proteins and the emulsifying salts. According to Carić et al. (1985), emulsifying salts are used in process cheese to remove calcium from the protein system, hydrate, peptise, swell, solubilise, and disperse the proteins, emulsify the fat and stabilise the emulsion, control pH and stabilise it, and contribute to the formation of an appropriate structure of the processed cheese after cooling.

Concerning the functions of the emulsifying salts listed above, it may be assumed that the differences in firmness of the process cheeses under study were related to a number of factors such as protein-protein interactions and/or reactions of proteins with the SE, C, and T commercial Joha emulsifying salts.

Proteolysis

Electrophoretic patterns of cheese bases I and II and young and mature Cheddar cheeses are shown in Fig. 2A. It may be observed that α- and β-casein in cheese base I remained intact after two weeks. In cheese base II, which contained added Savorase-A, β-casein was extensively hydrolysed after 2 weeks whereas α-casein was hydrolysed to a lesser degree. Tamime et al. (1990) reported that the extent of protein hydrolysis (expressed as glycine equivalent in mg/mL of the supernatant) in 2 weeks old cheese base II and mature Cheddar cheese was 400 and 490, respectively, and the soluble nitrogen content of the same cheeses was 1.06 and 1.02%, respectively. Since the maturity index of natural cheeses is measured indirectly by the level of glycine equivalent and soluble nitrogen contents, it may be concluded on this basis that the extent of proteolysis in cheese base II was similar to that in a mature Cheddar cheese (Law, 1987).

Casein fractions in the processed cheeses under study are shown in Fig. 2B (fresh samples), Fig. 2C (3-month storage at 10°C), and Fig. 2D (3-month storage at 30°C). The following phenomena have been observed:

(a) The two main bands of α-casein in the experimental processed cheese made solely from cheese bases I and II appear to be intact in contrast with the control processed cheese where α-casein has been extensively hydrolyzed. It is probable that firmness of the experimental cheese (Fig. 1A) could have been associated with reduced hydrolysis of α-casein and the presence of a greater amount of β-casein which has originated from cheese base I (Fig. 2A). (b) The concentrations of the β-casein fraction in the eight processed cheese blends examined when fresh and after storage for 3 months at 10°C and 30°C, appear to be similar. (c) In processed cheese blends A, B, C, and D, there was a progressive reduction in the intensity of the α-casein band which could have affected the firmness of these cheeses (Fig. 1A). A similar pattern may also be observed in blends A1, B1, C1, and D1. The increased number of fractions beyond α-casein could also play a role in the protein-protein interactions in relation to the firmness of the products. As shown in Fig 1B, the firmness of blend A1 was the highest (4.263 N in the sample stored for 3 months at 30°C).
It would be difficult to conclude that the firmness of the processed cheese was attributed only to the degree of casein hydrolysis and the possible interactions of the casein fractions. Probably, whey proteins also play a role. A yield of protein greater than would be expected from a normal cheese-making process was obtained when a cheese base was made from ultrafiltered milk (Ernstrom et al., 1980). This increased yield has been attributed to the retention of greater amounts of whey proteins in the cheese blend. It is assumed that during the melting stage of the cheese ingredients in the processing kettle at temperatures above 70°C, β-lactoglobulin unfolds due to denaturation (de Wit, 1985) and readily reacts with κ-casein as a result of disulphide interchange (Walstra and Jenness, 1984). This may explain the consistent difference in firmness between the experimental and control processed cheeses. It was also observed that as the amounts of cheese bases I or II in the processed cheese blend were reduced, and, thus, the amount of β-lactoglobulin was decreased, the products became softer (Fig. 1).

**Effect of Emulsifying Salts**

Another factor, which may affect firmness of processed cheese, is the type of the emulsifying salt used. The exact composition of the SE, C, and T emulsifying salts is not known, but according to the supplier in the UK (M. Nightingale - personal communication), the main components are sodium polyphosphates. Table 4 provides some specifications of the emulsifying salts used. According to the review by Carič et al. (1985), all condensed polyphosphates hydrolyze partially during cheese melting and the hydrolysis continues in the processed cheese during storage.

**Table 4. Some specifications of Joha emulsifying salts**

<table>
<thead>
<tr>
<th>Type change</th>
<th>Change Creaming</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4-P10</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE XXX (+) 0.2-0.4 XX - 50 Tr 45</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C XXX (-) 0-0.6 0 - 5 - 95</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T X (+) 1.0-1.5 0 95 5 -</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

O: nil; X: slight; XX: medium; XXX: strong; Tr: trace.
P: phosphate polymer.
Processed Cheese Made from Skim Milk Powder Cheese Base

Firmness of all the processed cheese samples under study exhibited a similar pattern. They became firmer after 3 months of storage and the firmness was higher in samples stored at 30°C than in samples stored at 10°C. It is possible that the higher firmness found in samples stored at 30°C may be attributed to a more extensive hydrolysis of the polyphosphates at this temperature as compared to 10°C. This could lead to a calcium-induced interaction among the proteins and result in a harder product.

Lee et al. (1979, 1986) concluded that the nonsedimentable nitrogen (possibly soluble nitrogen) in the supernatant obtained by centrifugation of an aqueous extract of processed cheese was markedly increased when the amount of the emulsifying salts (Joha S4, K, and T) was increased from 0.42 to 3.33%. These results may confirm the effect of emulsifying salts on the peptisation of proteins during the manufacture of processed cheese. The soluble nitrogen content in all the processed cheese blends is shown in Table 5. Using an analytical method different from that used by Lee et al. (1979), the following observations were made: (a) all the processed cheese blends stored at 30°C for 3 months contained slightly higher concentrations of soluble nitrogen than the freshly made processed cheeses, and (b) all processed cheese samples stored at 10°C for 3 months had lower soluble nitrogen contents. The former finding confirms that a more extensive hydrolysis of condensed polyphosphates takes place in processed cheeses during storage, particularly at a higher temperature. This effect may be considered similar to the effect of an increased amount of emulsifying salt in the processed cheese blend as reported by Lee et al. (1986).

**Microstructure**

Electron microscopy showed that there were marked differences in the microstructure of the raw materials used to produce the processed cheese samples. Cheddar cheese, which was made from full-fat nonhomogenised milk, consisted of a protein matrix in which large fat globules and their clusters were dispersed (Fig. 3) in agreement with the findings of other authors (Green et al., 1981). Because fat was extracted with chloroform from Cheddar cheese samples destined for the SEM examination, void spaces indicate in the micrographs the initial presence of the fat particles and their aggregates in the samples. Residues of the fat globule membranes may usually be seen in the voids.

**Table 5.**

<table>
<thead>
<tr>
<th>Processed Cheese</th>
<th>Duration of Storage</th>
<th>Fresh</th>
<th>10°C</th>
<th>30°C</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.538</td>
<td>0.494</td>
<td>0.576</td>
<td></td>
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<tr>
<td>Experimental</td>
<td>0.342</td>
<td>0.269</td>
<td>0.392</td>
<td></td>
</tr>
<tr>
<td>Blend A</td>
<td>0.362</td>
<td>0.341</td>
<td>0.398</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.498</td>
<td>0.443</td>
<td>0.531</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.441</td>
<td>0.414</td>
<td>0.459</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>0.539</td>
<td>0.474</td>
<td>0.550</td>
<td></td>
</tr>
<tr>
<td>A_1</td>
<td>0.553</td>
<td>0.469</td>
<td>0.571</td>
<td></td>
</tr>
<tr>
<td>B_1</td>
<td>0.576</td>
<td>0.480</td>
<td>0.575</td>
<td></td>
</tr>
<tr>
<td>C_1</td>
<td>0.469</td>
<td>0.460</td>
<td>0.478</td>
<td></td>
</tr>
<tr>
<td>D_1</td>
<td>0.541</td>
<td>0.479</td>
<td>0.560</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 3. Microstructure of Cheddar cheese.**

A: Void spaces in the protein matrix indicate the locations of fat globules (F) and their clusters in young Cheddar cheese. B: Mesophilic lactic streptococci (b); arrows point to residues of fat globule membranes which became noticeable after fat was extracted from the samples in preparation for SEM.
Fig. 4. Microstructure (SEM) of cheese bases I and II. 
A: Cheese base I (free of added proteolytic enzyme) had a compact structure. 
B: Cheese base II (made with added Savorase-A) had an open structure as the result of the proteolytic activity of the added protease.

Fig. 5. Microstructure (TEM) of cheese bases I and II. 
A: Cheese base I (free of added proteolytic enzyme) consisted of a compact protein matrix (p); 
B: Cheese base II (made with added Savorase-A) had an open protein structure (p) as the result of the proteolytic activity of the added protease.

spaces, particularly at a higher magnification (Fig. 3B). In addition, clusters of mesophilic lactic streptococci which originated from the starter culture were also present in the protein matrices (Fig. 3B) of the samples under study. There was a low incidence of calcium phosphate crystals in both Cheddar cheese varieties.

In contrast to Cheddar cheese, cheese bases I and II consisted of a protein matrix in which no fat was noticeable (Figs. 4 and 5). This was in agreement with the composition of the cheese bases which were made from reconstituted skim milk powder. There were differences between the structures of both cheese bases. Cheese base I was compact (Figs. 4A and 5A) but cheese base II was porous or open (Figs. 4B and 5B) as the result of the treatment with Savorase-A which had been added in order to stimulate accelerated ripening in this cheese base.

Processing resulted in the development of structures in the control and experimental cheeses (Fig. 6) which differed from the structures of the raw materials (Figs. 3, 4, and 5). As the result of emulsification, fat particles were reduced in dimensions from those in excess of 1 μm in Cheddar cheese to 0.322±0.105 μm (mean diameter ± standard deviation of the fat particle sections) in processed cheese blend A1, and to 0.362±0.144 μm, 0.336±0.127 μm, and 0.291±0.082 μm in processed cheese blends B1, C1, and D1, respectively. The emulsified fat particles were of globular
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Fig. 6. Control (A, B) and experimental (C, D) processed cheese samples stored for 3 months at 10°C (A, C) and 30°C (B, D).

Fat particles (as indicated by the void spaces) were relatively uniformly distributed in the protein matrix. In B and D, some fat particles were of irregular shapes (*). b: Streptococci; Small arrow points to crystalline inclusions; Large arrow points to a compact lump of cheese base.

shape in fresh processed cheese samples and were more or less uniformly distributed in their protein matrices.

Although the structures of the raw materials (Cheddar cheese, cheese base I, and cheese base II) used to make these processed cheeses considerably differed from each other, processing resulted in the development of structures in the processed cheese blends which were, in general, similar, irrespective of the proportions of the individual ingredients used. This similarity was particularly clearly evident in processed cheeses stored at 10°C (Figs. 6A and 6C). In all the processed cheeses stored at 30°C, the fat particles were of irregular shapes (Figs. 6C and D). Areas consisting of compact protein free of fat particles were occasionally seen in the experimental cheese (Fig. 6D) made with anhydrous milk fat and the cheese bases prepared from reconstituted skim milk. Either the cheese base proteins were not yet properly peptised when processing was arrested, or a proper emulsification of the anhydrous milk fat was more difficult to achieve than the emulsification of fat present in the form of fat globules in natural cheeses such as Cheddar cheese in the control processed cheese made under similar conditions.

Differences between the structure of the experimental processed cheese samples stored at 10°C and 30°C are more noticeable in TEM micrographs (Figs. 7 and 8). The irregular shapes of the fat particles may be the result of coalescence of smaller fat particles and an incomplete restoration of the globular shapes. However, the irregular shapes of some fat particles, particularly those shown in Figs. 8B and 8F (arrows), seem to indicate that additional emulsification of the larger fat particles probably took place during storage at the higher temperature. Image
Fig. 7. TEM of experimental processed cheese stored for 3 months at 10°C (A) or 30°C (B). Deformation of fat globules (F) is evident in the product stored at 30°C; arrows point to newly developed fatglobule membranes.
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**Fig. 8. TEM of processed cheese blends stored for 3 months at 10°C or 30°C.**

Processed cheese blend A1 stored at 10°C (A) and at 30°C (B); blend B1 stored at 10°C (C) and at 30°C (D), and blend D stored at 10°C (E) and at 30°C (F). Light arrows point to fat particles of irregular shapes, dark arrows point to newly developed fat globule membranes. b: Bacterium; c: Crystals of melting salt.

**Fig. 9. SEM of processed cheese blend A (A), blend B (B), and blend C (C).**

Granularity is well developed in A (arrows point to accumulated fat particles), is negligible in B, and is fine in C.

Analysis (approximately 200 fat particles per field) failed to show any statistically significant differences between the dimensions of the fat globule sections in cheeses stored at 10°C and 30°C. Rather than an increase in the dimensions of the fat particles, which would be anticipated in the case of coalescence, there was a slight tendency toward the reduction in the fat particle dimensions in one half of the samples shown in Table 6.
Some differences observed in the microstructures of the cheeses under study could be attributed to the type of cheese used in the blend (e.g., Cheddar cheese and cheese base I or II, or a combination of both cheese bases) in addition to the storage temperature. Processed cheeses made from cheese base I and II consisted of compact protein matrices possibly due to the removal of calcium ions from the casein micelles under the sequestering or calcium-complexing action of the emulsifying salts. The main differences in the microstructures of these cheeses as compared with the control cheese may be summarised as follows:

(a) Blend A, where all the young Cheddar cheese had been replaced by fat-free cheese base I and an amount of anhydrous milk fat making it equivalent to the fat deficient in cheese base II had a structure which, following storage at 30°C, upon freeze-fracturing gave an impression of granularity, as relatively compact protein areas were surrounded by areas rich in fat particles (Fig. 9A). The relatively high proportion of cheese base I in comparison with the proportion of mature Cheddar may also be a factor contributing to that granularity. (b) Blend B, which was made from young and mature Cheddar cheese and contained smaller proportions of cheese base I and anhydrous fat than blend A, had, when stored at 10°C, a less grainy structure (Fig. 9B) than blend A. (c) Blend C, in which all mature Cheddar cheese was replaced by cheese base I was macroscopically heterogeneous but the samples examined by SEM (Fig. 9C) and TEM showed finer granularity than blend A. The granular appearance was not noticeable in blend D where Cheddar cheese was only partially replaced. (d) In the remaining blends, i.e., A1, B1, C1, and D1, the microstructures were similar to the control and experimental cheeses, but blend A1 had a crumbly and sticky texture as revealed by sensory evaluation, probably because it consisted of a high proportion of young Cheddar cheese and cheese base II in which proteolysis was induced by added Savorase-A.

Various salt crystals were observed in the protein matrices of the raw materials and the processed cheeses under study (Fig. 10). It would be difficult to suggest the exact nature of these crystals because of lack of data regarding the components of the Joha emulsifying salts. However, it may be assumed that these crystals were phosphate complexes, possibly calcium phosphate, because the Joha salts contain a large proportion of sodium phosphate. Crystal formation in processed cheese has been reported in the literature when a known type of emulsifying salts has been used (Brooker et al., 1975; Rayan et al., 1980; Kaláb, 1981; Carić et al., 1985; Brooker, 1987; Carić and Kaláb, 1987; Pommert et al., 1988; Savello et al., 1989) and such data could be used to help identify the crystals observed in the present study.

This study indicates that the microstructures of the different blends of processed cheese were affected by the type of cheese (Cheddar and/or cheese base) used in the blend. It is safe to conclude that the new method used for the production of cheese bases I and II from reconstituted skim milk powder is suitable for the manufacture of block type processed cheese, which in this study has been marked as the experimental batch.

In processed cheese blends containing different amounts of Cheddar cheese and cheese base I or II, however, some undesirable characteristics, e.g., crumbliness or grainy structure, were observed. These could be eliminated by altering the processing conditions, e.g., by using different types of emulsifying salts.
Acknowledgments

Mr. M.F. Younis is indebted to the Egyptian Government for financial support. The authors thank Mr. A. Slack (Imperial Biotechnology Ltd.) for providing the Savorase-A sample, Dr. G. Stanley (Eurozyme Ltd.) for providing the starter culture, Dr. H.W. Modler for useful comments, and Mrs. Paula Allan-Wojtas and Miss Gisèle Larocque for skillful technical assistance. Image analysis was carried out by Mr. E. F. Bond. Electron Microscope Centre, Research Branch, Agriculture Canada in Ottawa provided facilities. Contribution 848 from the Food Research Centre in Ottawa.

References


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Taneya, Thomas MA. (1977). The Walstra 1',


Discussion with Reviewers

D. Paquet: What effects had anhydrous milk fat on the texture or microstructure of processed cheese?
Authors: The overall impression was that in the processed cheese blends it had no evident effect on the texture or microstructure.

D. Paquet: What type of mix do you recommend for the manufacture of Egyptian processed cheese?
Authors: Practically most of the blends were suitable for the Egyptian market. However, in order to reduce cheese imports to Egypt, the experimental cheese, which was made totally from cheese bases I and II, may be used to manufacture processed cheese locally.

D.N. Holcomb: Can the authors provide additional information on the emulsifying salts used, e.g., whether phosphates or citrates were used?
Authors: You have asked a very interesting question because the properties of the processed cheese are influenced by the constituents of the emulsifying salts used, e.g., phosphates or citrates. However, Joha SE, C, and T are commercial formulations of emulsifying salts which are widely used in the processed cheese industry. The exact formulation and the available technical data are, as a consequence, somewhat limited. The supplier had provided us with some information as shown in Table 4.

D.N. Holcomb: During the manufacture of cheese base, the reconstituted skim milk powder was ultrafiltered, diluted with water, and then diafiltered. It might be useful if the authors would explain why these two filtration steps were both necessary.
Authors: The two filtration steps were necessary for the following reasons: (a) the ultrafiltration was carried out primarily to concentrate the protein in milk and (b) the diafiltration process helped to reduce the level of lactose in the retentate.

D.N. Holcomb: Could the authors provide more detail of the staining procedure with uranyl acetate and lead citrate?
Authors: Thin sections were stained with a saturated uranyl acetate solution in methanol, stained with alkaline lead citrate (Reynolds, 1963) for 5 min, washed with methanol, and then dehydrated.

J. Heertje: Creaming time is considered to be an important variable in the production of processed cheese. No clear mention is made about the creaming time. Was it used to induce changes in the firmness? If so, were differences in microstructure observed?
Authors: It is a well established fact that creaming time can induce considerable changes in the firmness of processed cheese. In the present study, the creaming time was maintained at a constant period (i.e., using vacuum for 1 min at 85-90°C followed by stirring for 2 min) in order to minimise any variation between the processing of each blend.

J. Heertje: Can an explanation be offered for the considerable increase in firmness of the products after storage at 30°C? Is this reflected in the microstructure?
Authors: Differences in the firmness of fresh (soft) and stored (firm) cheeses cannot be explained on the basis of the micrographs obtained. The aggregation of fat particles and their fusion led to an increase in the fat particle dimensions. Large fat particles are usually found in soft processed cheeses (Shimp, 1983). Since the experimental cheese under study was firmer after storage at 30°C than the fresh cheese, we may assume that its high firmness is most probably associated with changes in the protein matrix.

Excessive heating is known to harden process cheese. It takes only several hours of heating at 82°C to severely thicken a processed cheese emulsion (Kalab et al., 1987).
The change in firmness is accompanied by the development of electron-dense areas in the heated processed cheese, but it is not known whether such areas consist of chemically modified protein or whether the protein is compacted in these areas.

Thinner sections and a high-resolution electron microscope would be necessary to reveal differences (if any) in the microstructures of the protein matrices of the soft and firm samples. Cryofixation of the cheeses followed by replication of planes obtained by freeze-fracturing and examination of the replicas by TEM would probably be even more suitable.

**I. Heertje:** Is there an indication in the protein phase for the existence of string-like structures as observed by others in processed cheese?

**Authors:** String-like structures similar to those shown by Taneya et al. (1980), Heertje et al. (1981), and Čarić and Kaláb (1987) were noticeable in the processed cheeses stored at 30°C (Fig. 11A); the incidence of minute electron-dense particles, assumed to be artefacts, would weaken any conclusions which we would attempt to draw on a possible relationship between the firmness of the processed cheeses and the ultrastructure of their protein matrices. The development of the artefacts has been quite difficult to avoid.

In addition to the string-like structures in the protein matrices, membranes were seen at a higher magnification in thin sections of process cheeses stored at 10°C to cover the emulsified fat globules (Fig. 11B).

**Fig. 11. High-magnification TEM of the A₃ processed cheese blend.**

**A:** String-like structures (large light arrows) were present in the protein matrices of processed cheese blends stored at 30°C; sample A₃ is shown in this micrograph as an example.

**B:** Membranes (large arrows) covering emulsified fat globules were found in processed cheese blends stored at 10°C. Small arrows in A and B point to minute electron-dense particles considered to be artefacts.
POLYMORPHISM OF TRIGLYCERIDES
A CRYSTALLOGRAPHIC REVIEW

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Abstract

In order to understand the role of fat in food systems detailed knowledge about the arrangement of triglyceride molecules is useful. The triglyceride molecules in a fat can be packed in alternative ways, each crystal form having different melting points. This phenomenon is called polymorphism. Based mainly on X-ray studies (single-crystal, powder diffraction, scattering) and Raman spectroscopy the structure of the different polymorphic forms, α, sub-α, β1, β2 and β, are explained. The emphasis is on work done in the author’s laboratory.

There is a close relationship between the polymorphic forms which is persistent also in the liquid state - the molecules are arranged two and two in a bilayer. The differences between the polymorphic forms are due to: (i) the hydrocarbon chain packing, (ii) the tilt of the chains versus the methyl end group plane, and (iii) differences in the methyl end group region.

From a technical point of view the polymorphic transitions of fats is of the highest importance. This paper describes a mechanism behind the \( \beta' \rightarrow \beta \) transition - the chain mobility in the methyl end group region causes this transition.

Introduction

The functional properties of fats are very important in many food systems. The type of dispersion, the solid-liquid ratio and the occurring crystal form often have a strong influence on the behaviour of the entire system. The triglyceride molecules in a fat can be packed in the solid state in alternative ways, each crystal form having different melting points. This phenomenon is called polymorphism. The evolution of the present knowledge of the polymorphism in triglycerides can be studied in detail in various articles [2, 4, 6, 9, 15-19, 21, 22, 24, 25, 28]. Up to this date no other triglyceride crystal structure than the \( \beta \)-form is completely determined [15, 19]. Since all the different polymorphic forms of fats occur and influence the physical properties of various food systems, knowledge about the structure of triglycerides in the different crystal forms is important.

The present review deals with the polymorphism of triglycerides - the structure of the different forms and the transitions between the forms.

Materials and Methods

The triglyceride samples used in the different studies were either purchased or prepared by standard methods [cf. 9]. Crystals for single-crystal work were grown from acetone at 20°C. A Weissenberg camera was used for the X-ray single crystal work, a diffraction-pattern-versus-temperature (DPT) camera [9, 27] was used to study the polymorphic transitions. The DPT camera was also used for the powder-diffraction and the scattering studies. The X-ray films were examined with a photodensitometer. Raman spectra were recorded with two different systems: A Cary 82 spectrophotometer and a Dilor RT1 30 Laser Raman with triple monochromator, both systems using Argon laser for excitation.

Polymorphic transitions of triglycerides

Triglycerides are known to crystallize in three typical polymorphic forms namely \( \alpha \), \( \beta' \) and \( \beta \). Fig. 1 shows a somewhat more complex picture of the polymorphic transitions. The two \( \beta' \)-forms are quite similar which will be discussed below. The sub-\( \alpha \)-form is normally not obtained, since very low temperatures are needed. As indicated in the figure...
Complete picture of the polymorphic transitions of triglycerides. All transitions are irreversible except the \( \alpha \rightarrow \text{sub-}\alpha \) transition. The dotted lines indicate the possibility of a direct crystallization in other forms than the \( \alpha \)-form. The possibility of growing crystals from solvents is also indicated [9].

All polymorphic forms can be obtained directly from the liquid state except the sub-\( \alpha \)-form. So far only the \( \beta_1 \)- and \( \beta \)-forms have been obtained from solvents. The transition liquid \( \rightarrow \alpha \rightarrow \beta_2 \rightarrow \beta_1 \rightarrow \beta \) is the complete way for triglycerides to find the optimum packing of the molecules. In a fat both chain length and degree of unsaturation can vary a lot but the same polymorphic forms are obtained. The main difference between different fats or between different pure simple or complex triglycerides is how fast the transition goes. As indicated in Fig. 1 the transitions need not to follow the complete way.

The chain packing of hydrocarbons

Regardless which kind of functional group that is present, all hydrocarbon chains in long chain compounds are packed in one of a few possible ways. The best method to describe the chain packing is to use the subcell corresponding to the smallest repetition unit within the chain layer. All subcells have been discussed thoroughly in a review by Abrahamsson and coworkers [1]. In simple triglycerides only three different subcells occur: the hexagonal \((h)\) subcell \((\alpha)\); the orthorhombic \((O.L)\) subcell \((\beta_2, \beta_1 \) and sub-\( \alpha \)) and the triclinic \((T//)\) subcell \((\beta)\).

The chain orientation

Based on X-ray powder diffraction Clarkson and Malkin [5] proposed that two acyl chains in a triglyceride molecule are arranged adjacently and the third one points in the opposite direction. This principal structure of triglycerides is illustrated in Fig. 2a. The hydrocarbon chain can then be close packed according to the different subcells mentioned above but still be orientated according to the principal packing in Fig. 2a. The molecules are packed in a way that two "chair like" molecules build up a dimeric unit. The bilayer structure illustrated in Fig. 2a consists of four triglyceride molecules or two dimers.

In 1948 Lutton [23] proposed an additional structure - the triple chain layer which is seen in Fig. 2b. This structure can occur when one specific chain in a triglyceride differs in length by four or more carbons. The triple chain layer also occur if one specific chain is unsaturated, i.e., 1,3 di-stearoyl-2-oleoyl-glycerol (common in cocoa butter).

In Fig. 3 a schematic comparison between \( \alpha \), \( \beta_1 \) and \( \beta \) is illustrated. The structures are in three planes showing both the close relation of dimeric units building up bilayers and the difference in hydrocarbon close packing. The dimeric units are in fact more or less consistent even in the liquid state.
POLYMORPHISM OF TRIGLYCERIDES

TEMP

Proposed structure of triglycerides in the liquid state [10].

Figure 4. Proposed structure of triglycerides in the liquid state [10].

Liquid state

Based on X-ray technique and Raman spectroscopy [10,16] a proposed structure of triglycerides in the liquid state has been developed. In the liquid state the triglycerides are arranged in the chain-shaped conformation (see Fig. 4) and most often the dimeric unit is present. The model is dynamic, i.e., both size and orientation of the lamellar units vary with diffusion rates of the molecules. When the temperature is increased the size of the lamellar units decreases. The degree of order within the lamellar units, however, is proposed to be almost constant.

When the temperature of a melt is decreased the lamellar units are increased in size until crystallization finally takes place. Formation of a crystal nucleus can be regarded as the limiting point when formation of adjacent all-trans chains within the lamellar unit dominates over gauche conformation.

The α-form

Fig. 5 shows the α-form [12] of triglycerides. The proposed structure is based on evidence from X-ray diffraction and Raman spectroscopy studies. The chain axes are indicated as lines, since the main part of the hydrocarbon chains is oscillating. The molecules are arranged perpendicular to the methyl end group plane and the hydrocarbon chains are hexagonally (H) close packed.

From a molecular packing point of view the α-form is unsatisfactory because of the irregular methyl end group region. Due to this irregularity the hydrocarbon would be expected to possess a high degree of mobility. This mobility, together with the hydrocarbon chain oscillation, normally induces a rapid transformation from the α-form to one with better chain packing.

Figure 5. Proposed structure of the α-form of triglycerides projected along a short axes. The chain axes are indicated; the main part of the hydrocarbon chains is oscillating and hexagonally close packed; the methyl end group regions are somewhat more disordered, as in liquid crystals [12].

The β-forms

Fig. 6 shows the main features of the proposed β1-form of the triglyceride triundecanoin. The structure has been derived from a single crystal study [12] which has been recently further extended [8]. Measurements of the (ab) lattices in the β1- and β-forms indicate that the glycerol group region in the β1-form can have the same structure as in the β-form. If the α-form is compared with the β1-form it can be seen that the methyl groups are better packed in the β1-form. This is due to a tilt of the hydrocarbon chains in relation to the methyl end group plane. The hydrocarbon chains are arranged according to the orthorhombic (O.L) subcell.

The a-c projection of the β1-form is closely related to the a-c projection of the β-form [cf. Fig. 7]. The main difference between the β1 and β-forms is seen in the two other projections (see Fig. 3).

The β1-form shows two directions of chain tilt in the c-b projection. This change in tilt is assumed to take place at the methyl end group plane. A second β-form, β2, of the triglyceride triundecanoin has been observed [12]. It differs from the β1-form in its alignment in the different planes. The β2-form is probably tilted only in the c-a plane and vertical in the c-b plane.

The β2-form

Fig. 7 shows the crystal structure of trilaurin [19]. The β-form is in fact the only polymorphic form so far to have its structure completely determined.
The proposed structure of the \( \beta' \)-form of triundecanoin [8]. The glycerol part is proposed to be arranged in the same way as in the \( \beta \)-form of trilaurin [cf. 12].

The chains are tilted in relation to the methyl end group planes in the c-a projection as well as in the c-b projection (see Fig. 3). In the \( \beta \)-form the hydrocarbon chains are arranged according to the triclinic T\( _{11} \) subcell.

The \( \beta' \rightarrow \beta \) transition

From a technical point of view the \( \beta' \rightarrow \beta \) transition is most important. When producing margarine it is important that the fat crystals are stable in the \( \beta \)-form. This is, however, not always the case. If, e.g., hydrogenated rapeseed oil with low content of erucic acid (LOBRA) is used the \( \beta \)-form is developed very rapidly [7]. As a consequence the texture of the margarine becomes unacceptable due to the presence of large crystals [29]. This rapid \( \beta' \rightarrow \beta \) transition can be hindered by using additives like diglycerides [11, 14] or sorbitan tristearate [20], plant breeding [13, 26] or just by blending the hydrogenated LOBRA oil with other oils.

When the \( \beta' \rightarrow \beta \) transition occurs, the methyl end group planes of two adjacent bilayers need to slide in a way that all the bilayers are tilted in the same direction. This transition is strongly dependent upon the structure of the methyl end group plane. Consequently some triglyceride molecules give a stable \( \beta' \)-form while others are rapidly transformed to the \( \beta \)-form.

**Figure 6.** Proposed structure of the ca-projection of the \( \beta' \)-form of triundecanoin [8]. The glycerol part is proposed to be arranged in the same way as in the \( \beta \)-form of trilaurin [cf. 12].

**Figure 7.** Molecular arrangement in the \( \beta \)-form of trilaurin [19].

**Figure 8.** Comparison between the proposed structures of the \( \beta' \)-form of triundecanoin (a) and tridecanoin (b). Shaded areas indicate a higher degree of hydrocarbon chain mobility.

Fig. 8 shows the proposed structure of the \( \beta' \)-form of triundecanoin and tridecanoin. The \( \beta' \)-form of tridecanoin is derived from the \( \beta' \)-form of triundecanoin by "cutting" one carbon from every acyl chain [8]. Furthermore the bilayers are adjusted in order to get a similar distance in the methyl end
group region as in the case of triundecanoin. The difference between the two triglycerides what concerns the methyl end group region is rather obvious. Tridecanoin shows "empty areas" - shaded in Fig. 8. This indicates a higher mobility in the case of tridecanoin. Since the $\beta' \rightarrow \beta$ transition involves sliding as mentioned above, tridecanoin consequently should undergo the $\beta' \rightarrow \beta$ transition faster com-pared with tridecanoin. This is also the case. In fact all simple triglycerides with odd acyl chains are stable in the $\beta'$-form and all even acid simple triglycerides are stable in the $\beta$-form.

The structures and polymorphic behaviour discussed above are based on investigations of simple saturated triglycerides. X-ray diffraction data, however, indicate that these structures are valid also for most complex triglycerides as well as mixtures of triglycerides. In a natural fat both the chains length and the degree of unsaturation can vary, but nevertheless the same polymorphic forms are obtained.

References


Discussion with Reviewers

P. Birker: The author proposes that triglyceride melts contain liquid crystalline regions, and that the size of these regions varies with temperature. This model is based on his own work published in reference 10, i.e., the observation of a very broad reflection in the diffraction pattern of e.g., liquid trimyr-ristin at about 22 A. I accept that this reflection can indicate liquid crystallinity but there is no proof that the size of liquid crystalline regions change with temperature. The observed difference (ref. 10) in half peak value of this reflection of 25 A at 50°C and 26 A at 60°C are not significant. What is the proof or experimental support for the proposed variation of size and orientation of or-dered regions with diffusion rates?
L. Hernqvist

**Author:** I agree that the difference in half peak values, 25 Å at 50°C and 26 Å at 80°C is small but nevertheless it has been observed. I suggest further investigations. If this difference is considered to be existing it must be due to a reduction of the lamellar units. The possibility of using line broadening to estimate the size of the domains in a L2 phase in a liquid crystalline phase was used 1983 (Fontell K, Hernqvist L, Larsson K, Sjöblom J (1983) On structural relations between lipid mesophases and isotropic reversed micellar (L2) solutions. J. Coli. Interface Sci. 93 453-460).

**P. Birker:** One of the main features of the β structure is the likely alternation of chain direction (ref. 8) either at the glycerol group or at the methyl end planes. This cannot be seen in the projection selected for Figure 6.

**Author:** The alternation of chain direction occurs in the c-b projection which is given in Fig. 3. In this figure the alternation is proposed to occur at the methyl end plane. The question as to whether the chain tilt alternates in the glycerol group region or at the methyl end plane cannot be unambiguously determined. In an earlier discussion of the β-form (ref. 16) the space groups was proposed to be P21;21c. The new single-crystal data (ref. 12), however, show that the earlier observed apparent orthorhombic symmetry was due to twinning, the correct space group is P21/c.

**J.W. Hagemann:** To my knowledge, there has been no conclusive evidence as to where the bend in β-forms occurs, whether at glycerol or at methyl groups. Larsson’s early findings suggested the glycerol region, as no chain tilt at the methyl gap was consistent with β-forms. Irregardless of where the tilt occurs, it is known that conversion to β is a cataclysmic event at the molecular level producing fat bloom in even chain lengths but not in odd chain lengths. The similarity of your structures in Fig. 8 would imply that there would be essentially no difference in the conversion between odd and even. The figure merely points out that evens will more readily convert because of voids in the methyl gap. There are also large differences between odds and evens in thermal behaviour and long spacing data which suggest different β and/or β-forms. If we assume that chain tilt occurs at the methyl gap, the lower group of molecules in Fig. 8 can be reversed to produce a packing, that does not contain major voids. Perhaps this problem could be more fully explained.

**Author:** Concerning the bend in the chain tilt, see answer given above to P. Birker. The alternation of chain tilt only exists in the c-b projection, not in the c-a projection. The presence of voids in the c-b projection of the β-form of tridecanoin (Fig. 8b) indicates that tridecanoin (an even triglyceride) easily can be transformed to the β-form. When the β → β transition occurs, the methyl end-group planes of two adjacent bilayers need to slide in order to get the same tilt in all bilayers, both in the c-a and the c-b projections. The importance of the methyl end-group plane has been pointed out earlier (Larsson K (1966) Alternation of melting points in homologous series of long-chain compounds. J. Am. Oil Chem. Soc. 43 559-562) and discussed in the case of β-forms (6).

**K. Sato:** Can you explain the difference in the subcell structure between β2 and β1? My opinion is that both forms are packed according to orthorhombic perpendicular subcell packing, but an arrangement of the subcell axis with respect to the basal plane may be different in relation to the different methyl end packing.

**Author:** I quite agree, in ref. 12 we explain this viewpoint more thoroughly.
Abstract

The size of flake-cut meat is an important quality determinant of comminuted meat products which, potentially, depends upon a large number of factors. Temperature and whether or not the meat is pre-broken have a major influence on the resulting particle size distribution, as does aperture size. Meat flaked at -7°C produced two to three times more flakes than at -3°C. Under some conditions the particles produced were as little as 0.4 mm thick and characteristically were thicker at one end.

High speed photography, used to visualise the cutting action, indicated that size reduction occurs in a controlled manner providing that the meat is neither too cold, nor too warm. Above -1°C the meat merely deforms rather than being cut.

Single, discrete particles, examined using scanning electron microscopy (SEM) and cryo-SEM, unexpectedly did not exhibit the usual features of cleanly-cut meat. The lack of ultrastructural detail was attributed to a smearing of sarcoplasmic fluid produced by a localised, transient rise in temperature during flaking.

Introduction

Consumer demand for conveniently sized, easy-to-cook, no-waste portions of consistent quality, coupled with the need to utilise the lower quality cuts of the carcass, has led to a proliferation of comminuted meat products. One of the primary reasons for comminuting is to overcome the connective tissue toughness associated with many of these lower quality cuts. Consequently, one of the most important quality determinants is particle size, which, with associated characteristics of the particles such as shape and surface morphology, determines not only the subjective perception of particle size (Berry & Civile, 1986) but also influences the physical appearance of the product (Durland et al., 1982) and the adhesion between particles (Acton, 1972).

Flaking is probably the most recent method of comminution. A commonly used flaking system, introduced in the 1970's by the American company, Urschel Laboratories, is now widely used in the manufacture of grillsteaks and other restructured meat products. Unlike bowl chopping or mincing, the technique produces discrete particles (provided the meat is cold enough), which are amenable to particle size analysis and investigations of the surface characteristics of the particle.

As well as reporting upon the surface microstructure of the particles and factors influencing the size distribution of flake-cut meat, this paper is also concerned with understanding how size reduction is achieved - information which is invaluable for the development and control of new and existing products.

UK-style grillsteaks

The scheme on the right of Figure 1 shows the typical sequence of operations involved in the manufacture of grillsteaks in the UK. Meat is usually purchased as frozen blocks (typically up to 30 kg), which need to be tempered,
Sheard et al.

Fig. 1. Two common methods of manufacture of restructured meat products. The sequence of operations on the right is followed by most UK manufacturers; the scheme on the left has been followed by most of the literature on the subject.

usually between -1°C and -8°C, to facilitate subsequent processing. The required size reduction is normally achieved in two stages - a relatively coarse comminution procedure, pre-breaking, followed by a comparatively fine comminution procedure, flaking. Sodium chloride, either as crystals or as a solution, is added during mixing, together with any herbs, spices or other non-meat ingredients. Good adhesion between meat pieces in the cooked product is normally attributed to proteins solubilized and extracted during this procedure. The most popular means of imparting shape to the resulting sticky mass is via a high speed patty former.

The scheme on the left of Figure 1 is typical of most of the literature on the subject. In commercial practice in the U.S., however, the meat is usually hydro-flaked in the frozen state and then thawed or tempered to ≤4°C before being flaked (Berkowitz, personal comm.). Alternatively the frozen meat may be thawed to about +2°C and then flaked. There is, therefore, an important difference between this and common practice in the UK, where the meat is usually flaked in a semi-frozen condition. As in the UK, patty forming machines are used by most manufacturers of restructured meat products, though some small processors will form the finished product as a log - a practice declining because of cost considerations.

Products made for the USA market, usually referred to as restructured steaks, tend to be sold to hotels, restaurants and institutional establishments, rather than through retail outlets as in the United Kingdom.

Our studies have concentrated on meat flaked below the initial freezing point (ifp) as this is more relevant to UK manufacturers.

Flaking using the Comitrol processor

Meat is cut by impelling it at high speed, typically 3,000 rpm in the UK, or 3,600 rpm in the USA, against a stationary cutting head (Figure 2). There are a large variety of cutting heads available, differing in the number of cutting stations and the aperture size (i.e., opening size) (Figure 3). Providing that the meat is hard enough, typically 2°C or 3°C below the initial freezing point of meat (about -1°C), discrete pieces are produced; the appearance of meat flaked above the ifp is quite different, superficially resembling meat which has been ground (Jolley & Purslow, 1988), as shown in Figure 4. The temperature at which there is a transition from the production of discrete flakes to that of mince-like strands depends upon aperture size. Mince-like strands may be produced at several degrees below freezing, if the aperture size is too small. We have observed strands from meat flaked through a 1.5 mm aperture at a temperature of about -4°C.

Measuring particle size in comminuted meat products

There are many situations where size measurements are desirable. Consequently, a wide range of particle sizing techniques have been developed over the last twenty years or so, particularly for measurements in the sub-micron range, where current interest is keenest. However, despite the relatively large
particle characteristics of flaked meat

Fig. 3. Diagram showing the design of the cutting head of the Comitrol® processor and its characteristic dimensions.

Fig. 4. Photograph showing appearance of meat flaked through an aperture size of 4.6 mm at a temperature of about -1°C.

Fig. 5. Particle size range for some common methods of measuring particle size.

number of techniques available (Fig. 5), and the universal recognition of the need to measure particle size in comminuted meat products, relatively few studies of particle size in relation to meat comminution have been reported.

One exception to this generalization is the work of Girard et al. (1985) who used a Coulter Counter® to show the change in the size distribution of bowl chopped meat during comminution for up to 40 minutes. Particles ranged in size from 1 to 30 microns, and since the diameter of a muscle fibre is typically about 50 microns (Light et al., 1985), this implies that tissue disruption must have been quite extensive. However, the technique requires the sample to be highly diluted, which could cause further tissue breakdown.

Various microscopical techniques have been used in size analysis, but, in order to give statistically significant data many individual particles need to be measured, with the result that manual procedures are generally slow and labour intensive. These disadvantages have largely been overcome with the advent of automated image analysis techniques. The more sophisticated of these machines can perform projected area measurements and measure statistical diameters such as the commonly used Feret and Martin diameters. Holes can be recognised and routine shape analysis performed. Images may be stored, together with derived data, which may be presented numerically or graphically.

Automated image analysis has been used for example to measure the fat to lean ratio in boneless fresh, or cured, meats (Newman, 1984) and minced meat (Newman, 1987), and also for quantifying the amount of collagen, elastin and bone in histological sections of meat (Hildebrandt & Hirst, 1985).

Thus the technique applies equally well to images produced by scanning electron or transmission electron microscopes and direct images of larger objects.

Factors potentially affecting particle size

Using a video image analysis (VIA) technique we have investigated some of the large number of factors which may determine particle size. Our results to date are summarised in Table 1 (Sheard et al., 1989, 1990; Sheard et al., unpublished data).

Of the factors listed under the heading "Machine Parameters" there are four which are pre-selected by the user (aperture size, number of cutting stations, impeller speed and impeller design). In a recent study we made size measurements of meat flaked at speeds of 3,360 and 6,680 rpm through aperture sizes of 1.5, 6.1 and 19.0 mm using two different types of impeller. At least two flake heads, differing in the number of cutting stations, were examined for each aperture size. Aperture size was the most important determinant of particle
Table 1. Factors potentially affecting the particle size of flaked meat.

<table>
<thead>
<tr>
<th>Machine parameters</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Aperture size.</td>
<td>Correlates with particle diameter; also affects particle thickness.</td>
</tr>
<tr>
<td>2. Number of cutting stations.</td>
<td>Probably affects particle thickness.</td>
</tr>
<tr>
<td>3. Impeller speed (3,000 - 7,000 rpm)</td>
<td>Probably not important.</td>
</tr>
<tr>
<td>4. Impeller design.</td>
<td>Affects machine operation (noise, flake head wear) but probably not critical for particle size.</td>
</tr>
<tr>
<td>5. Flake head wear.</td>
<td>Less efficient cutting; increased heat gain.</td>
</tr>
<tr>
<td>6. Feed rate.</td>
<td>No published data.</td>
</tr>
</tbody>
</table>

Properties of meat raw materials

1. Temperature of meat. Meat flaked at low temperatures produces particles which are thinner and have a greater overall surface area. Mince-like strands are produced if the meat is too warm. Input piece size limits maximum size obtainable; meat pre-broken by grinding breaks up easily. Small differences observed between lean beef, turkey breast and turkey thigh. No published data.

2. Input piece size; pre-breaking.


4. Type of muscle.

5. Other factors e.g. animal age, PSE and DFD meat, post-mortem history, freezing rate.

size, influencing the size distribution of particles, the number of particles per gram of sample, the resulting surface area per gram of sample, the mean particle diameter and also the mean thickness of flakes. There was little effect on particle size of impeller speed or the design of the impeller. For a given aperture size and temperature, the mean particle thickness was also dependent upon the number of cutting stations, more cutting stations giving rise to slightly thinner particles.

As with any other comminution system the cutting surfaces of the machine become worn over a period of time, particularly if the meat is flaked at temperatures well below the initial freezing point or if the material contains a high proportion of gristle, or bone. We have observed that, for given conditions, a damaged flake head, as shown in Figure 6, tended to produce mince-like strands at a slightly lower temperature than a new head. Also there was a greater amount of residual material left on the inner surface of the head after flaking for approximately the same period of time. Analysis of this residue showed a slightly higher connective tissue content than that of the original raw material, suggesting that some connective tissue had been stripped out during flaking and implying some destruction of structure rather than clean cutting.

Of the factors investigated to date the two most important properties of the raw material are temperature and whether or not it has been pre-broken. The practical consequences of using meat which is either too cold or too warm have been summarised by Bezanson.
properties of the meat, which becomes more brittle with decreasing temperature (Munro, 1983). The change in mechanical properties with temperature could simply be due to the ice content of the meat at a given temperature. To investigate the relationship between temperature and particle size, measurements were made of the number and thickness of particles for diced meat (19 mm cubes) flaked, without being pre-broken, through an aperture size of 12.9 mm at 1°C intervals between -2 and -7°C (Fig. 7). The relationship between ice content and temperature in this range is highly non-linear. The number of particles per gram of sample increased from about 40 at -2°C to 80 particles/g at -4.5°C. Between -4.5°C and -7°C, the number of particles increased from about 80 to 140 particles/g. The ice contents at -2 and -4.5°C are 48% and 73%, respectively and 78% at -7°C (Morley, 1972). The mean particle thickness decreased from about 1.4 mm at -2 to 0.8 mm at -4.5°C and to 0.5 mm at -7°C. The surface area, which is inversely related to the thickness, ranged from 2,100 mm²/g at -7°C to 780 mm²/g at -2°C. It is extremely doubtful that a change in ice content of just 5% would account for these marked changes in particle size between -4.5°C and -7°C. The data appear, therefore, to substantiate our earlier suggestion that factors other than ice content - such as the increased viscosity of the unfrozen liquor and the dehydration of the fibres - determine the size of comminuted meat (Sheard et al., 1989).

**Table 2. Means (± standard deviation) of number and thickness of particles for meat flaked through aperture sizes of 6.1, 9.9 and 19.0 mm at -3°C, with and without pre-breaking, and -7°C.**

<table>
<thead>
<tr>
<th>aperture size (mm)</th>
<th>number of particles per gram</th>
<th>thickness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-3°C (pre-broken)</td>
<td>-7°C</td>
</tr>
<tr>
<td>6.1</td>
<td>164 ± 29</td>
<td>368 ± 37</td>
</tr>
<tr>
<td>9.9</td>
<td>61 ± 13</td>
<td>148 ± 29</td>
</tr>
<tr>
<td>19.0</td>
<td>30 ± 3</td>
<td>108 ± 6</td>
</tr>
</tbody>
</table>

1 meat introduced to the machine as bandsawn sticks approximately 15 x 3.5 x 3.5 cm.

(1975) and demonstrated in semi-quantitative fashion by Ellery (1985). Pre-breaking hard frozen meat produces excessive fines, is detrimental to product cohesion and can cause equipment failure.

Table 2 shows the effects of the temperature of the meat and pre-breaking on the size of particles obtained from the flaking operation (Sheard et al., 1990). Pre-broken particles ranged in size from tiny fragments of less than 1 mm in diameter to large, irregularly-shaped pieces approximately 4-5 cm in diameter. For a given aperture size, meat which had been pre-broken at -7°C then flaked at -7°C had two to three times the number of particles per gram compared with meat which had been pre-broken at -3°C and then flaked at -3°C. Furthermore, at -3°C, far fewer particles were produced where the meat had not been previously pre-broken. Particle thickness was also highly dependent upon temperature, but dependent upon aperture size to a lesser extent. At a given aperture size, particles produced by flaking at -7°C were about half as thick as those flaked at -3°C.

These results have important implications for the textural quality of restructured meat products. For many particles, it is likely that the fibres are cut obliquely, but let us consider the two limiting cases, where the fibres run parallel, or at 90 degrees, to the long axis of the flake. In the first case, some flakes may be as little as 8 fibres thick (i.e. 0.4 mm, assuming a fibre diameter of 50 microns); whilst in the second case, fibres may be only 0.4 mm long. Since restructured products are required to simulate the eating quality of whole muscle steak (where steaks are typically cut transversely to the fibre direction, to give a thickness of 1-2 cm), many fail to meet this objective because the constituent particles are too small to confer sufficient fibre character.

In broad terms the effect of temperature can be explained on the basis of the mechanical properties of the meat, which becomes more

**Flaking single pieces of meat**

In the discussion which follows we use the term 'cutting', though flaking need not necessarily involve a true cutting action. A crack, for example, could be initiated by impact at the surface and could propagate through a piece of meat without the cutting edge traversing the width of the piece. Fracture might occur, therefore, by a combination of impact, cutting and other mechanisms. The term is used here because, visually, the
surfaces created resemble those of cleanly-cut meat or meat that has fractured in a brittle fashion. Such surfaces are usually smooth and when placed in juxtaposition, generally fit back together again quite readily. In meat which is not cut cleanly, or meat which exhibits viscoelastic fracture behaviour, distended and twisted strands of connective tissue and groups of fibres can be seen protruding from a relatively rough, uneven surface (Dobraszczyk et al., 1987).

Figure 8 illustrates how a single piece of meat (approximately 3 x 2 x 0.5 cm) is cut. Each piece was flaked individually at about -3°C through an aperture size of 40.6 mm. The resultant flake-cut pieces were collected and the original piece reconstructed. It can be seen that in each case the piece has been cut in a radial fashion regardless of fibre direction, giving rise to a number of wedge-shaped pieces, each having a thick end and a thin end. Each piece was cut in a similar way, regardless of fibre direction and the presence or absence of fat, as would occur with a true cutting action.

Meat deforms less readily against the cutting head at low temperatures (Anon., 1980). Consequently the depth of cut is reduced producing thinner particles, as indicated in Figure 7. However, meat at low temperatures (≤-3°C) is also more brittle and 'cutting' could be accompanied by multiple cracking, which might also account for the greater number of particles observed at reduced temperatures (Table 2).

High speed photography has played a crucial role in design modifications to the impeller (Koberna, personal communication). Figure 9 shows three different types of impeller. The first generation of impellers were straight-bladed, which, because of their noisy operation were superceded by impellers whose blades either sloped backwards or forwards (Fig. 9A). At high feed rates, however, meat could rub against non-cutting surfaces causing an unnecessary rise in temperature. This particular problem was overcome by using a dog-leg impeller (Fig. 9B); but this had the disadvantage that excessive wear occurred in a relatively small area of the flaking head. The latest generation of impellers, the dio-cut impeller (Fig. 9C), have a ring of alternately forward and backward sloping blades which moves the meat across the entire length of the
cutting head thus minimising wear, without the disadvantages incurred with previous impeller designs.

We have used high speed photography to show how the cutting action depends upon aperture size, temperature and whether or not the meat has previously been pre-broken. A single piece of meat (approximately 3 x 2 x 0.5 cm), flaked through an aperture size of 40.6 mm at a temperature of about -3°C, was reduced to flakes in about a third of a revolution (i.e. 0.005 s); however, several revolutions (0.14 s or more) were required to cut a piece of the same size using a 4.6 mm aperture. In this case, pieces of meat remained intact to the point of being cut by the sharpened surfaces of the flaking head. Meat which had previously been pre-broken by grinding, however, tended to deform, or even break up, on impact with the impeller.

The period of time required to reduce a piece of meat to flakes also depends upon temperature. Attempts to gauge the time required to "cut" an individual piece of meat at -1°C, however, were impracticable because of the difficulty in distinguishing one piece from subsequent pieces falling into the cutting head. However, for a given aperture size, the overall cutting time may well be ten times or more at flake temperatures above the initial freezing point compared with lower temperatures (-3°C) where discrete flakes are produced. Above the ipf, rather than being cut cleanly, the meat is deformed as it is forced against the flaking head, becoming smeared around the inside surface by the impeller.

The time required for cutting is indicative of the type of fracture behaviour. Under some conditions, several hundred flakes may be produced from a piece of meat of the size observed using high speed photography and, thus, to make a single cut (i.e. to propagate a single crack) across the face of the specimen, must take place more rapidly than the overall cutting time. Munro (1983) has shown that under brittle conditions, crack propagation in meat, over a distance of about 10 mm, takes less than 0.02 s; under viscoelastic conditions, fracture occurs more slowly, over 2-3 s. Regardless of the temperature of the meat, the rotational speed of the impeller is constant. Thus, for a flaking head having twenty cutting stations, the average time required to traverse a single cutting station is 0.001 s, which is far too short to allow for crack propagation under viscoelastic conditions. Consequently, the meat merely deforms, rather than being cut cleanly, as occurs at lower temperatures when the meat is brittle.

**Ultrastructure of single flakes**

Great advances have been made, particularly over the last 20 years, in elucidating the hierarchical structure of muscle and the ultrastructure of muscle fibres subject to a variety of pre- and post-rigor treatments. These morphological features have been described and reviewed by many authors (Voyle, 1979; Offer & Trinick, 1983; Lewis et al., 1986). Microscopical analyses have also been used to show the location, appearance and structure of the major components in a wide range of meat products (Lewis, 1979; Theno et al., 1978; Voyle et al., 1986). Many studies have been undertaken on a variety of comminuted meat products to resolve whether or not the matrix could be accurately described as a true meat emulsion (Swasdee et al., 1982; Foegeding, 1989; Regenstein, 1989). However, there have been few microscopical studies undertaken on restructured meat products, and the studies that have been made have concentrated on the finished product, either frozen (Nusbaum et al., 1983; Bernal & Stanley, 1986) or cooked (Cardello et al., 1983; Bernal & Stanley, 1986), rather than the morphology of individual flake-cut pieces.

Cardello et al. (1983), who examined products, after cooking, made from meat flaked through aperture sizes of 1.5, 19.0 and 40.1 mm suggested that fibres (even those of meat flaked through a 1.5 mm aperture) appeared similar to "normal" cooked whole muscle tissue. Bernal and Stanley (1986) commented that there was evidence of "a damaged fibrous structure" in the cooked product. They did not report the conditions under which the product was made, however. Nusbaum et al. (1983) were interested primarily in the effects of freezing rate on the microstructure of the product and, in particular, its relationship with quality and cooking losses. These workers suggest a mechanism to explain why slowly frozen products, with large ice crystal cavities, should have greater cooking losses.

Unfortunately, the processing conditions

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**Fig. 9. Photograph illustrating three different designs of impeller.** A) Impeller with 3 backward sloping blades. Pieces of meat slide away from the base of the impeller and are cut at the trailing edge. B) Dog-leg impeller. Meat is cut in the V-shaped pocket. C) Dio-cut impeller having alternately forward and backward sloping blades. The backward sloping blades are shortened so that the meat slides away from the base and into the path of the forward sloping blade behind it.
Fig. 10. Edge view of a single flake of turkey breast flaked through an aperture size of 4.6 mm at -3°C, showing the characteristic wedge shape of particles, and their typical thickness. The upper view has been constructed from 4 separate electron micrographs; the two lower views show the ends of the flake. The thick and thin ends measure 1.88 mm and 1.19 mm, respectively. The overall length of the flake is about 12 mm. Notice also the alignment of surface material from right to left, suggesting that cutting begins at the thick end.

were not sufficiently similar, or well documented, to make comparisons between the three studies, or to draw any definite conclusions.

Figure 10 is an edge view of a single flake at low power magnification, illustrating the typical wedge shape apparent in many flakes. The width of the thick and thin ends, obtained using the caliper facility on the microscope, were 1.88 and 1.19 mm, respectively. These are the kind of values we expect from our VIA results. There also appears to be some orientation of surface material from right to left, suggesting that cutting begins at the thick end of the flake, an observation confirmed by examination of a large number of flakes. This finding, together with the result that individual pieces appear to be cut in a radial fashion (Fig. 8), suggests that individual cuts made on a single piece of meat terminate, rather than begin, at the focus. The cuts are presumably made in a sequential fashion.

Flakes were collected immediately after flaking, fixed in glutaraldehyde, dehydrated, critical point dried and sputter coated with gold. Certain characteristic features were observed in material prepared in this way. Ice crystal cavities were obvious; these ranged in size from about 25 to 75 microns; fibre direction was also obvious in most flakes (there did not appear to be any preferential fibre direction in those flakes which were examined). In some flakes, individual fibres could be seen, as could strands of perimysial connective tissue. However, the most striking feature was the lack of fine structure normally associated with cleanly-cut meat at magnifications up to 650 times.

Figure 11 is a micrograph of meat cut perpendicular to the fibre direction with a sharp scalpel, examined at a magnification of 80 times. It illustrates the well-known morphological features of meat structure, viz, individual fibres organised into fibre bundles. Endomysial and perimysial connective tissue can be seen surrounding individual fibres and fibre bundles, respectively. Figures 12 and 13 show the typical appearance of the flakes we obtained, cut along (Fig. 12) and across (Fig. 13) the fibres. The flakes were collected and prepared for microscopy as previously described.

The disorganised web of fibrous matter seen on the surface of the specimen in Fig. 12, which obscured the underlying array of meat fibres, could be collagenous material. This interpretation, however, may be ruled out for two reasons. All the flakes were examined at both high and low magnifications, and any thick tracts of connective tissue or large areas of collagenous material would have been evident at lower magnifications. Secondly, of the flakes examined, which included turkey breast, turkey thigh and forequarter beef, none were found which displayed the characteristic fibrous structure of cleanly-cut meat, though in some cases the surface appearance was rather more amorphous than the matted appearance shown in Fig. 12. Fig. 13 differs superficially from Fig. 12 because of the different fibre directions; it also differs in that the surface appearance is more amorphous. More importantly, neither specimen, regardless of fibre direction, exhibited the characteristic detail associated with cleanly-cut meat.

The relatively smooth surface of the
Figure 12 a, b. SEM micrograph of turkey thigh at two different magnifications. The structure (f) is a muscle fibre running parallel to the plane of the surface, with a diameter of about 55 microns. Notice the absence of cross-striations, even at the highest magnification. The nature of the fibrous material which is evident elsewhere is less clear. The specimen in Fig. 12, and also the fact that the ends of fibres were not twisted and distorted are also indicative of a clean cutting action. Similar surface characteristics have been observed for specimens that have failed in tension at low temperatures (≤15°C) and high strain rates, where the meat behaves in a brittle fashion (Dobraszczyk et al., 1987). One would expect a quite different appearance for meat which was not cut cleanly.

The appearance of the flakes could be explained in several ways. It is known that the temperature rises during comminution and some of the ice melts (Ellery, 1985; Sheard et al., 1990), thus making water available for dissolution of already concentrated solutes, which could become deposited on the freshly created surfaces of cut fibres. Alternatively, the appearance could be due to the denaturation of heat labile proteins. Although the overall rise in temperature of the meat during flaking is only a few degrees, because of the high latent heat of freezing of water, this represents a considerable change in enthalpy, and the transient rise in temperature could be substantially higher, particularly at the surface of a flake, where, presumably, most heating takes place.

Fig. 13. SEM micrograph of turkey breast flaked through an aperture size of 4.6 mm at -3°C, showing the largely amorphous appearance of the cut surface. Ice crystal cavities range from about 25 to 125 microns, and suggest the fibre direction to be at 90° to the plane of the surface. The fissure, running diagonally across the micrograph, may denote the boundary between fibre bundles or a gap opened up between adjacent fibres.

A third possible explanation of this surface phenomenon is related to the residue that collects on the inside of the flaking head, small amounts being picked up as the particles emerge from the apertures. We must also accept the possibility, of course, that these observations were an artefact arising from the preparative procedures. However, by examining flakes using cryo-SEM, where artefacts resulting from fixation, dehydration and critical point drying are avoided, it becomes possible to confirm whether or not these earlier observations were valid.

Figure 15 shows the typical appearance of a specimen examined in this way. The raw material was collected in liquid nitrogen as it
Fig. 14. SEM micrograph of pork Longissimus dorsi cut at about -4°C using a bandsaw. The cut was made perpendicular to the fibre direction at about 130 mm from the anterior end of the muscle. Individual fibres bundles are readily apparent; some individual fibres can be distinguished, but any fine detail is lacking. The micrograph was obtained using a Jeol JSM-35 SEM. Courtesy E. Beatty, University College, Cork.

Fig. 15. Cryo-SEM micrograph of beef semitendinosus flaked through an aperture size of 4.6 mm at -2.5°C. The fissure, running from the bottom left hand corner, probably denotes the boundary between fibre bundles. Strands, probably of perimysial connective tissue, can be seen bridging the two surfaces. The general appearance suggests that fibres run at 90° to the plane of the surface.

Fall from the flaking head, thus preserving any ice still present. Individual flakes were then transferred to the SEM cold stage whilst still frozen, and then coated with gold and observed as before.

Ice crystal cavities were generally smaller, presumably because of the rapid freezing immediately after flaking, whilst individual fibres were more readily identifiable. Again, specimens had an amorphous appearance, similar to those seen previously, suggesting that the appearance of flakes shown in Figures 12 and 13 are, indeed, genuine observations.

Conclusion

These microscopical observations of individual particles, together with the cine film and VIA results, provide a powerful insight into the mechanism by which semi-frozen meat is cut during flaking. Based upon cine film records and other qualitative observations, individual pieces of meat appear to be cut in a controlled fashion provided that the meat is cold enough to prevent it merely deforming rather than being cut, but not so cold as to cause multiple cracking.

SEM micrographs of these cut surfaces revealed little of the structural detail normally associated with cleanly-cut meat, possibly because it was obscured by a surface smearing of sarcoplasmic fluid. Further characterization of the surface morphology would be of value, as would an examination of the microstructure of material flaked above the ifp. Scanning confocal microscopy, to examine the sub-surface ultrastructure, may prove a useful complementary technique to SEM.

Particle size measurements, made under a wide range of practically relevant conditions, have been made and these will enable the manufacturer to identify which factors are most likely to influence particle size, and by how much. This information is essential not only to reduce variability in product quality arising from differences in particle size, but also in designing the textural quality of new products.

High speed photography provides an invaluable tool in visualising exactly what happens during flaking. The technique could be applied equally well to other systems, not only as a research tool, but also for design purposes and as a diagnostic tool.

The information gained from studies of this type are essential to predict from first principles, rather than trial and error, how changes in the raw material or machine parameters will affect the level of size reduction achieved, and the reasons for variability in response.

Acknowledgments

We are grateful to Eddie Beatty, of University College, Cork, for permission to include Figure 14. The figure comes from work undertaken at UCC by Paul Jolley, who gratefully acknowledges the financial support of the Kellogg Foundation.
We are also grateful to Urschel Laboratories for their co-operation and helpful advice. Figures 2 and 3 are taken with permission from Facts, Flakes & Fabricated Meats. (1980) published by Urschel Labs. Inc., Valparaiso, USA.


References


**Discussion with Reviewers**

J.T. Clayton: Particle size is not adequately defined. Does it mean an overall measure of size (volume)? Or is there one dimension (thickness) that is overriding in importance?

**Authors:** For all but the simplest shapes, size is, in fact, very difficult to define. A sphere, for example, can be defined by a single dimension - the radius - but this cannot be said of irregularly shaped particles. Many sizing techniques assume that particles are spheres. Moreover different techniques do not necessarily measure the same property of the particle. One would not expect, therefore, VIA to give the same result as a Coulter Counter, for example, which expresses size as the diameter of a sphere having the same volume as the particle.

It would be misleading to suggest that one dimension is of overriding importance. A single flake (i.e. a thin, broad particle) can be conveniently thought of in terms of just two dimensions - its thickness and diameter - but this would not tell us if the projected surface of the particle was circular or rectangular or if its outline was ragged or smooth. One should also remember that comminution will always result in particles of different sizes. The object, therefore, in particle size analysis is usually to arrive at a size distribution which is usually in terms of weight or diameter or some other property depending on the technique being used.

R.A. Segars: How is 'surface area' of the flakes defined, e.g. does surface area times thickness = volume?

P.J. Lillford: The authors mention that the particles are often wedge shaped. In view of this they should state how particle thickness was measured. Are the values quoted a mean thickness or the maximum thickness of particles?

**Authors:** For flaked particles the available surface area is important for protein extraction. It is, therefore, a sensible property to measure. VIA measures the projected surface area and, assuming a constant thickness, the product of the surface area and thickness does give an approximation of the volume. Ignoring the area at the 'edges' the available surface area is approximately double the projected surface area.

Using VIA it is possible to calculate a mean thickness from the total projected surface area. This gives a mean value for the whole population of particles but, clearly, this is an oversimplification. As already pointed out, particles are usually thicker at one end. Moreover our observations indicate that the larger particles are also thicker but we have no data on how the thickness varies within a population of particles.

C.J. Scott: Impeller speed affects length of cut; aperture size affects width. Table 1, however, implies impeller speed is not important.

**Authors:** We appreciate that aspect ratio (i.e. the ratio of the width of a flake to its length) is an important property of a particle and one which, conceivably, will depend upon the material being flaked and the conditions employed during flaking.

According to our results meat flaked at higher speeds had a greater projected surface area (but not significantly so), implying that the particles were thinner. However, as we did not specifically measure aspect ratio we cannot confirm or deny the reviewer’s comments.

P.J. Lillford: The aperture size quoted in Table 2 refers presumably to the width of the cutting orifice. Why should the width of the orifice affect particle thickness?

**Authors:** The thickness of the flakes produced will depend upon the extent to which it protrudes into the apertures of the cutting head. This can be best understood by considering a single, spherical piece of meat, as shown below. The arrows denote the relative motions due to the impeller and centrifugal force.
Porosity and specific gravity were determined in Blue cheeses made from (A) homogenized 14% fat pasteurized cream standardized to 3.9% fat with pasteurized skim milk, (B) homogenized 14% fat raw cream standardized to 3.9% fat with raw skim milk, and (C) pasteurized milk (3.9% fat). There were no significant differences between cheeses made from (A) and (B) in porosity and specific gravity. Cheeses made from (A) and (B) had greater porosity, lower specific gravity and more, but smaller holes than cheese made from (C). The negative linear relationship between porosity and specific gravity, and the insignificant differences in fat and moisture content in cheese made from (A), (B) and (C) suggest that many of the holes contained air and not whey.

Introduction

The presence of holes containing air in Blue cheese is very important. They enhance the necessary carbon dioxide-air exchange during mold growth and enhance the development of the fruiting structure of Penicillium roqueforti. Blue cheese made from homogenized milk or homogenized cream recombined with skim milk appears to have an open body and a porous structure that favors mold growth and sporulation (Morris, 1981).

In a previous paper we measured porosity, specific gravity and fat dispersion in Blue cheeses made from various homogenized mixtures of nonfat dry milk, water and butter oil, from pasteurized cream and skim milk and from raw cream and skim milk (Kebary and Morris, 1988). One of the reviewers of that work, Dr. M. L. Green, suggested that the contents of the holes (pores) in the cheese might be determined. We did not have sufficient compositional data to do this calculation at that time. Therefore, this paper is an attempt to estimate the amount of air in Blue cheeses made from variously treated milks.

Materials and Methods

Cheesemaking

The following materials were used in cheesemaking (sources are indicated in parenthesis): milk (3.9% fat; Mid-American Dairymen, St. Paul, MN); starter (Chr. Hansen’s Laboratory, Inc., Milwaukee, WI); calcium chloride and Penicillium roqueforti powder (Dairyland Food Laboratories, Inc., Waukesha, WI). 920 kg of milk was separated and the cream and skim milk divided into two portions each. One portion of each cream and skim milk was pasteurized at 72.2°C for 15 seconds. The other portion was used as separated. The 14% fat creams were double-stage homogenized at 13700 + 3448 kPa (2000 + 500 psi) in a Gaulin Model 125183MF12A, 384 L/H (Manton Gaulin Company, Inc., Everett, MA). The homogenized 14% fat pasteurized cream and raw cream were standardized to 3.9% fat (the fat content of the beginning milk) with pasteurized skim milk and raw skim milk, respectively. Another lot of the 3.9% fat milk was pasteurized at 72.2°C for 15 seconds. Blue cheeses were made from 454 kg lots of the homogenized mixtures and a 182 kg lot of unhomogenized pasteurized milk by a method described by Morris (1981), except that calcium chloride was added to pasteurized mixtures. Based on previous

Key words: Blue cheese, porosity, specific gravity, air content.
Tables 1 – 5. The porosity, stated as a ratio of the total area of holes to the area of the cheese observed (Table 1), mean of holes area in cm² (Table 2), number of holes (Table 3), specific gravity (Table 4), and air content in percent (Table 5) of Blue cheeses made from: (i) homogenized 14% fat pasteurized cream standardized to 3.9% fat with pasteurized skim milk (sample A); (ii) homogenized 14% fat raw cream standardized to 3.9% fat with raw skim milk (sample B); and (iii) pasteurized milk (sample C).

Table 1: Porosity

<table>
<thead>
<tr>
<th>Sample</th>
<th>Trial 1ᵃ</th>
<th>Trial 2ᵃ</th>
<th>Mean</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.0808</td>
<td>0.0796</td>
<td>0.0802*</td>
<td>0.0006</td>
</tr>
<tr>
<td>B</td>
<td>0.0765</td>
<td>0.0737</td>
<td>0.0751*</td>
<td>0.0014</td>
</tr>
<tr>
<td>C</td>
<td>0.0590</td>
<td>0.0560</td>
<td>0.0575**</td>
<td>0.0015</td>
</tr>
</tbody>
</table>

* = not significantly different;
** = significantly different.

Table 2: Mean of Holes Area (cm²)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Trial 1ᵃ</th>
<th>Trial 2ᵃ</th>
<th>Mean</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.1225</td>
<td>0.1250</td>
<td>0.1238*</td>
<td>0.0013</td>
</tr>
<tr>
<td>B</td>
<td>0.1225</td>
<td>0.1250</td>
<td>0.1238*</td>
<td>0.0013</td>
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<tr>
<td>C</td>
<td>0.2549</td>
<td>0.2574</td>
<td>0.2562**</td>
<td>0.0013</td>
</tr>
</tbody>
</table>

Table 3: Number of Holes

<table>
<thead>
<tr>
<th>Sample</th>
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<th>Trial 2ᵃ</th>
<th>Mean</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>75</td>
<td>72</td>
<td>74*</td>
<td>1.5</td>
</tr>
<tr>
<td>B</td>
<td>71</td>
<td>67</td>
<td>69*</td>
<td>2.0</td>
</tr>
<tr>
<td>C</td>
<td>26</td>
<td>25</td>
<td>26**</td>
<td>0.5</td>
</tr>
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</table>

Table 4: Specific Gravity

<table>
<thead>
<tr>
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<th>Trial 2ᵃ</th>
<th>Mean</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.0427</td>
<td>1.0438</td>
<td>1.0433*</td>
<td>0.0006</td>
</tr>
<tr>
<td>B</td>
<td>1.0453</td>
<td>1.0457</td>
<td>1.0455*</td>
<td>0.0002</td>
</tr>
<tr>
<td>C</td>
<td>1.0489</td>
<td>1.0495</td>
<td>1.0492**</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

Table 5: Air Content (%)

<table>
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<tr>
<th>Sample</th>
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<th>Trial 2ᵃ</th>
<th>Mean</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.0036</td>
<td>0.0030</td>
<td>0.0033*</td>
<td>0.0003</td>
</tr>
<tr>
<td>B</td>
<td>0.0032</td>
<td>0.0028</td>
<td>0.0030*</td>
<td>0.0002</td>
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<tr>
<td>C</td>
<td>0.0027</td>
<td>0.0020</td>
<td>0.0024*</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

experience in making Blue cheese, lot sizes of these magnitudes do not influence structure. Cheesemaking trials were done in duplicate.

Materials and methods for determination of porosity and specific gravity were the same as described by Kebary and Morris (1988), except that porosity was calculated from four photographs for each trial, and specific gravity was determined using 10 cubes of cheese for each trial. Porosity is a ratio of the total area of holes and the area of the cheese (photograph) observed (Kebary and Morris, 1988). Specific gravity was measured based on a density gradient system using varying solutions of kerosene and monochlorobenzene (Stoll, 1966).

Moisture content of cheese before salting was determined using moisture/volatiles tester type SAS (C.W. Brabender Instrument, Inc., Hackensack, NJ). Fat content was determined using the Wopojnner fat test (Case et al., 1985). The equation to determine specific gravity of milk (Jenness and Patton, 1959) was modified to calculate the amount of air in cheese as follows:

\[ S = \left( \frac{F + N + W + \text{air}}{\left(\frac{F}{S_F}\right) + \left(\frac{W}{1}\right) + \left(\frac{N}{S_N}\right) + \left(\% \text{ air}/S_{\text{air}}\right)} \right) \]

where \( S \) = specific gravity; \( F \) = fat, \( N \) = solids not fat; \( W \) = water; \( S_F = 0.93; S_N = 1.6007; S_{\text{air}} = 0.0012 \). All parameters were determined or are given except \% air. The equation was solved for \% air.

Results and Discussion

The porosity of Blue cheeses made from homogenized 14% fat pasteurized cream standardized to 3.9% fat with pasteurized skim milk (A), homogenized 14% fat raw cream standardized to 3.9% fat with raw skim milk (B), and pasteurized 3.9% fat milk (C) was measured. Blue cheeses made from homogenized 14% fat cream standardized with skim milks (A and B) were more porous and significantly different (\( P < 0.05 \)) from cheese made from unhomogenized milk (C; Table 1 and Fig. 1), as postulated by Morris (1988). Cheeses made from (A) and (B) had more but smaller holes and were significantly different (\( P < 0.05 \)) from cheese made from (C) (Tables 2 and 3, and Fig. 1). Cheeses made from homogenized products (A and B) were not significantly different from each other (\( P > 0.05 \)) in porosity, number of holes and the mean of the area of holes (Tables 1, 2 and 3, and Fig. 1).

Cheeses made from the homogenized products (A
Porosity, Specific Gravity and Air Content in Blue Cheeses

Figure 1. Light photomicrograph of sections of Blue cheeses made from homogenized 14% fat pasteurized cream standardized to 3.9% fat with pasteurized skim milk (A), homogenized 14% fat raw cream standardized to 3.9% fat with raw skim milk (B), and pasteurized milk (C). F - fat; H - holes; P - protein matrix.

and B) were not significantly different (P greater than 0.05) from each other in specific gravity. Cheese made from (C) had higher specific gravity and was significantly different (P less than 0.05) from cheeses made from A and B (Table 4). Analysis of data from the cheeses shows that the most porous cheese (A) had the lowest specific gravity and the least porous cheese (C) had the highest specific gravity (Tables 1 and 4, and Fig. 2).

Cheese made from A, B, C were not significantly different (P greater than 0.05) from each other in moisture which were 45.4, 45.5 and 45.6%, respectively, and in fat content which were 22.5, 22.6 and 23.2%, respectively.

Cheese C had lower air content than cheeses A and B (Table 5) which were more porous and had lower specific gravity, but these differences did not reach the statistically significant level (P greater than 0.05). While cheeses A and B were more porous and had lower specific gravity than cheese C, cheeses A, B and C were not statistically different (P greater than 0.05) in moisture and fat content which might suggest that many holes contained air and not whey.

In conclusion, cheeses made from homogenized products had more porous structure, lower specific gravity, more (but smaller) holes than cheeses made from unhomogenized pasteurized milk. The negative linear relationship between porosity and specific gravity and the insignificant differences among cheeses in fat and moisture content might suggest that most of the holes, particularly in cheeses made from homogenized products, contained air and not whey.
K.M.K. Kebary and H.A. Morris

Acknowledgement

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References


Editor's Note: All of the reviewer's concerns were appropriately addressed by text changes, hence there is no Discussion with Reviewers.
AN ENZYME/SURFACTANT TREATMENT AND FILTRATION TECHNIQUE FOR THE RETRIEVAL OF Listeria monocytogenes FROM ICE CREAM MIX

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Hummelstown, PA

Abstract

This study combines an enzyme/surfactant treatment with centrifugation and prefiltration to solubilize food constituents in a dairy product containing Listeria monocytogenes, remove the constituents by a second filtration and examine the isolated bacteria under the scanning electron microscope. Treatment of an ice cream mix with a combined 2\% (w/w) trypsin and 2\% (w/w) Tween 80 solution for 20 minutes at 35\(^\circ\)C resulted in proteolysis of the dairy mix without lysing the bacteria. Centrifugation at 4300 \(x\) g for 20 minutes at 5\(^\circ\)C concentrated the bacteria in the form of a pellet which was subsequently purified through a prefiltration system prior to a final filtration through a polycarbonate filter with 0.4 \(\mu\)m pores. The bacteria on the filter were fixed in a glutaraldehyde solution, postfixed with osmium tetroxide, dehydrated in an ethanol series, impregnated with hexamethyldisilizane and air-dried. This procedure made it possible to retrieve sufficient concentrations of purified bacteria on the filter for examination in the SEM.

Introduction

In the past, filtration techniques for the examination of dairy products have not been feasible for a variety of reasons. Primarily, dairy products and in particular, ice cream, are inherently viscous because of the presence of fat and casein micelles making filtration virtually impossible. Nutting et al. (1959) investigated the use of membrane filtration to select coliforms and pioneered the use of warmed surfactant (Triton X-100) added to ice cream to maximize its filterable volume. Sharpe et al. (1979) investigated a number of foods and the effects of dilution, temperature, pressure, enzymes and surfactants on their ability to be successfully filtered using hydrophobic grid membrane filter (HGMF) and the membrane filter (MF) techniques. Peterkin and Sharpe (1980) demonstrated the increased recovery of five common bacteria from ice cream using membrane filtration and incubation with a protease and surfactant. Entis et al. (1982) developed aPrefiltration, enzyme (trypsin) and surfactant (Tween 80) treatment prior to application of the HGMF technique for the enumeration of coliform bacteria from dairy products. They showed that the use of an enzyme/surfactant technique was not deleterious to the cells as measured by the aerobic plate count. The above enzyme/surfactant methods have been applied to dairy products to remove interfering substances, allowing bacterial enumeration. Previous efforts by Amelang (1988, unpublished) demonstrated the need to apply this procedure to scanning electron microscopy to obtain "clean" images of bacteria growing in the ice cream mix.

Pasteurized dairy products other than fluid milk have relatively low numbers of microorganisms, <50,000/g (Anon., 1985) and no foodborne pathogens should be present in the finished product. Listeria monocytogenes has been isolated from milk and dairy products and is becoming increasingly well known as a dangerous food pathogen. Over 33 ready-to-eat dairy products, primarily ice cream and novelty items, have been recalled since 1985 because of contamination by L. monocytogenes. This organism can proliferate at refrigeration...
temperatures and survive at freezer temperatures; hence the reason for concern within the food industry.

This study utilizes the enzyme/surfactant treatment (Nutting et al. 1959; Sharpe et al., 1979; Peterkin and Sharpe 1980; and Entis et al. 1982) prior to the filtration and fixation necessary for observation of *L. monocytogenes* cells with the scanning electron microscope. Chemical toxicity was performed to ensure that the enzyme/surfactant treatment did not have a deleterious effect on the size or morphology of the cells. Three drying techniques: hexane, point drying (CPD), and freeze drying were compared to obtain the method which would yield clear micrographs. Morphological differences, which could occur when *L. monocytogenes* is grown under less than optimum conditions could lead to misidentification and under-reporting of this organism and therefore were investigated.

### Material and Methods

#### Preparation of Inoculum

*L. monocytogenes* strain Scott A was obtained from the culture collection of M. Doyle at the University of Wisconsin. The culture was maintained on trypticase soy agar supplemented with yeast extract (TSYE broth, BBL) slants at 4°C. Prior to use, a 10 ml tube containing trypticase soy broth supplemented with 0.6% yeast extract (TSYE broth, BBL) was inoculated by the loop method and allowed to incubate for 18 h at 35°C.

From an actively growing 18 h culture serial dilutions were made in potassium phosphate buffer (0.1% stock, pH 7.2, Bell et al., 1985) to obtain a concentration of approximately 10⁷ cells/ml. Ten milliliter cultures of the filter were added to 500 ml of ice cream mix and incubated for 10 h at 35°C to achieve logarithmic phase of growth. This suspension was then used for the enzyme/surfactant studies.

The ultra-high temperature-treated, unflavored ice cream mix was obtained from HP Hood (Boston, MA) and contained the following ingredients and concentrations (w/v): skim milk, 43.12%; cream, 43.93%; condensed skim milk, 5.35%; granulated sugar, 16%; stabilizer, 0.5% and emulsifier, 0.1%. It contained 14% milk fat and was stored frozen at -20°C prior to use.

#### Enzyme/Surfactant Treatment

The ice cream mix was treated with an enzyme, 2.0% w/w trypsin (Sigma Chemical Co., St. Louis, MO) and a surfactant, either 1.0% w/w Triton X-100 (Alkayly polyether alcohol, J.T. Baker Chemical Co., Phillipsburg, NJ) or 2% w/w Tween 80 (Polyoxyethylen sorbitan monooleate, Difco Laboratories, Detroit, MI) prior to filtration. Each 10 ml-treated sample was incubated for 20 min at 35°C and centrifuged at 4300 x g (Sorvall Super Speed RSC-6, I van Sorval Inc., Norwalk, CT) for 20 min at 5°C. The pellet was resuspended in 10 ml potassium phosphate buffer solution. A sample of the suspension was viewed under phase contrast microscopy (Zeiss Axioskop, Carl Zeiss, Inc., West Germany) to ensure the presence of the microorganism. Viable cells exhibiting the tumbling motility characteristic of this organism were evident. The sample was prefiltered using a 13 mm Prefilter Type AP25 (Millipore Corp., Bedford, MA) and the filtrate was subsequently passed through a 13 mm polycarbonate filter 0.4μm pore (Nucleopore Corp., Pleasanton, CA).

#### Preparation of Filters for SEM

After final filtration, the filters were transferred from the Skinny holders (Belman Sciences Inc., Ann Arbor, MI) to CPD specimen-processing capsules [BioRad Microscience, Cambridge, MA] and immersed in 3% glutaraldehyde (GA) in 0.15M sodium cacodylate buffer, pH 7.1 (Electron Microscopy Sciences-EMS, Ft. Washington, PA) for 1 h at room temperature. After removal of the glutaraldehyde and three washes in the same buffer concentration, the samples were postfixed in vapor saturated with 1% osmium tetroxide in 0.15M sodium cacodylate buffer, pH 7.1 (EMS) for 1 h at room temperature under a hood. The samples were then washed in distilled water, followed by dehydration in a gradient series of ethanol baths for 3 min each. Two methods of drying were used. One set of capsules was capped and transferred to a Polaron E3000 Critical Point Dryer (BioRad) and CO₂ (CP31.5°C and 7.5 X10⁶ Pa) was used as the final transitional fluid. The second set of capsules was treated with two 3 min baths of HMDS (EMS) and air-dried (AD) for 1 h. All filters were mounted onto aluminum stubs with double sticky tabs and a spot of colloidal silver and sputter-coated with 28 nm of gold in a Polaron (International Scientific Instruments- ISI, Pleasanton, CA). The prepared stubs were viewed using an ISI-60 Scanning Electron Microscope operated at an accelerating voltage of 10 kV and photographed using Polaroid 52 film (ISO-400).

#### Freeze Drying (FD) Method

A 10 ml ice cream mix sample was subjected to the enzyme/surfactant treatment, filtered and the filter was mounted onto an aluminum stub. The stub was initially placed onto a polished copper block cooled in liquid nitrogen and then transferred to a Virtis 15 SRC Sublimator (Virtis Company Inc., Gardiner, NY) and dried according to the manufacturer's instructions. Following sublimation, the stubs were gold coated and examined in the SEM.

#### Chemical Toxicity Study

The three chemicals (trypsin, Triton X-100, and Tween 80) were tested for detrimental effects on the growth or colony size of *L. monocytogenes* prior to use in any treatments. Samples of the culture grown in TSYE broth were incubated for 20 min at 35°C with two concentrations of each chemical (0.5 and 1.0% for Triton X-100, 1.0 and 2.0% for trypsin and Tween 80). The samples were subsequently serially diluted and plated onto the surface of TSYE broth containing 1.5% agar. After incubation for 48 h at 35°C, these plates were enumerated and colony sizes were measured. These experiments were performed in...
Enzyme/Surfactant-Filtration Technique for Ice Cream

Results and Discussion

Enzyme and Surfactant Treatment

Trypsin combinations with either Triton X-100 or Tween 80 were compared to determine the more suitable surfactant for this treatment. After initial screening, we concluded that the combination of 2.0% trypsin and 2.0% Tween 80 produced images with substantial breakdown of the unfilterable portions of the ice cream mix (i.e., fats and proteins). Figure 1 shows that this combination enhances the break-up and filtration and allows the recovery of a population of cells. Figure 2, a micrograph of a diluted but untreated suspension from preliminary work in this lab by J. Amelang, is evidence that without such treatment, isolation of the bacteria from the ice cream mix is impossible.

The enzyme/surfactant treatments supplement the work reported by Sharpe et al. (1979), Peterkin and Sharpe (1980), and Entis et al. (1982). Previous reports also use enzymes and surfactants prior to the filtering of foods to obtain bacterial cell counts. Our objective, however, was to solublize corpuscular food constituents, without damaging the bacteria, filter off the solution and examine the remaining bacteria by SEM. This method was performed more easily than previous procedures mentioned above because enzyme and surfactant were added directly to the ice cream sample avoiding the need for stock solutions and sample dilution. Using our method, applications for determining microbial counts by the plate count method or observations of cells by bright field could be performed. It is evident from the micrographs that the treatment is effective in breaking down the components of the ice cream mix while not affecting the quantity or quality of L. monocytogenes cells.

Freeze-Drying Method

We hoped that a freeze-drying method would circumvent the need for the conventional and time consuming fixing and drying procedures. Examination of Figure 3 showing a filter of a treated sample demonstrates the amount of unsolubilized components obscuring the bacteria and indicates that the additional baths of ethanol in the CPD or HMDS methods of drying actually aid in the further removal of those components.

Chemical Toxicity Study

Preliminary chemical toxicity experiments verified that trypsin, Triton X-100, and Tween 80 did not have a deleterious effect on the survival, colony size, or morphology of L. monocytogenes. It is evident from the data obtained in replicated experiments (Table 1) that there is no difference in total numbers and very little difference in colony sizes after exposure to these chemicals when compared with the untreated sample.

This finding correlates with the work performed by Entis et al. (1982) in which total bacterial cell counts from enzyme-treated and filtered foods were compared with filtered untreated foods. No decrease in numbers was obtained in the treated samples. Further support for survival of bacterial cells is illustrated.
in Figures 4-6 taken from plates incubated for 48 h at 35°C. Upon visual analysis, bacterial cell sizes and morphology appeared to be similar. We were curious to see if there were vast morphological size differences in the cells when grown in the ice cream mix at different temperatures. Christophersen (1973) raised questions about morphological changes that may or may not be evident in the bacterial cell
Enzyme/Surfactant-Filtration Technique for Ice Cream

Table 1. Total numbers and size of L. monocytogenes colonies when grown in TSYE broth and treated with varying concentrations of trypsin, Triton X-100, or Tween 80 for 20 min, at 35°C.

<table>
<thead>
<tr>
<th>Chemical Treatment</th>
<th>Concentration</th>
<th>Total Number&lt;sup&gt;1&lt;/sup&gt; (CFU/ml)</th>
<th>Size&lt;sup&gt;2&lt;/sup&gt; (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td></td>
<td>3.6 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>3.25</td>
</tr>
<tr>
<td>Trypsin</td>
<td>1.0</td>
<td>2.0 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>2.95</td>
</tr>
<tr>
<td>Trypsin</td>
<td>2.0</td>
<td>1.5 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>2.65</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>0.5</td>
<td>2.5 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>3.00</td>
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<tr>
<td>Triton X-100</td>
<td>1.0</td>
<td>1.5 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>2.95</td>
</tr>
<tr>
<td>Tween 80</td>
<td>1.0</td>
<td>2.6 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>3.25</td>
</tr>
<tr>
<td>Tween 80</td>
<td>2.0</td>
<td>3.1 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>3.25</td>
</tr>
</tbody>
</table>

<sup>1</sup>Total numbers are estimated from duplicate plates; CFU=colony forming units

<sup>2</sup>Size is represented as the mean of seven colony diameters.

Fig. 1. Ice cream mix containing L. monocytogenes grown for 12 h at 35°C. Sample treated with 2% trypsin and 2% Tween 80 for 20 min, passed through a prefilter and 0.4 μm polycarbonate filter. HMDS + AD. Bar=1 μm.

Fig. 2. Ice cream mix containing L. monocytogenes grown for 48 h at 21°C. Treatment included centrifugation and successive filterings (Whatman 49 filter paper and then, 5 μm and 0.45 μm polycarbonate filters). CPO. Bar=1 µm.

Fig. 3. Filter with treated sample of L. monocytogenes in ice cream mix grown for 7 d at 4°C and treated with 2% trypsin and 2% Tween 80. FD. Bar=1 µm.

Fig. 4. Untreated sample of L. monocytogenes grown in TSYE broth for 48 h at 35°C. Bacteria fixed on agar. HMDS + air-dried. Bar=1 µm.

Fig. 5. L. monocytogenes grown in TSYE broth for 48 h at 35°C and treated with 2% trypsin for 20 min. HMDS + AD. Bar=1 µm.

Fig. 6. L. monocytogenes grown in TYSE broth for 48 h at 35°C and treated with 2% Tween 80 for 20 min. HMDS + AD. Bar=1 µm.

Figs. 7, 8, 9: L. monocytogenes grown in ice cream mix, treated with 2.0% trypsin, and 2.0% Tween 80, pre-filtered and filtered through a 0.4 μm polycarbonate filter. CPO. Bar=1 µm.

Fig. 7. Culture conditions 7 d at 4°C.

Fig. 8. Culture conditions 12 h at 35°C.

Fig. 9. Culture conditions 15 h at 21°C.
grown under suboptimum temperature conditions. The observance of size differences or significant morphological changes from rods to cocci could indicate inadequacies in the testing of ice cream mix for the presence of pathogens, leading to possible misidentification, especially of L. monocytogenes. Because this is a major concern to the dairy industry as a whole, the samples were inoculated and kept at either 4°C, 21°C, or 35°C for the length of time needed for the cells to achieve logarithmic growth. The samples then underwent the previously described enzyme/surfactant treatment, centrifugation, prefiltration and fixation. All filters were then critical point-dried, gold coated and viewed in the SEM. Figures 7, 8, 9 show samples at all three temperatures. Measurements of the cells were taken from each figure and were compared using statistical analysis. The results indicate no significant size differences among the three temperatures although the samples grown at 35°C did appear smaller than those grown at the lower temperature.

Summary

The presence of L. monocytogenes in ice cream mix has been previously enumerated by plating techniques but because of the density of the mix constituents, microscopic viewing of the sample has been difficult. An enzyme/surfactant treatment with 2.0% trypsin and 2.0% Tween 80 when coupled with centrifugation and filtration yields micrographs of acceptable quality. While the quicker drying method using HMDS can be used to indicate recovery of cells, CDP remains the method of choice where size or morphological changes are concerned. These pretreatment and SEM preparative techniques can be easily used and do not have deleterious effects on the bacteria. The possibility of using SEM to answer microbiological questions in ice cream is now feasible.

Acknowledgement

We are very grateful to J. Amelang for his preliminary work with ice cream mix filtration. This article is authorized for publication as Paper No. 822B in the series of the Pennsylvania Agricultural Experiment Station.

References


Discussion with Reviewers

C.W. Donnelly: Are the methods developed applicable to retrieval of Listeria from other food systems, such as meats? What about tissue samples? If so, the methods could be extremely useful for human and animal epidemiological studies.

Authors: Although experiments addressing other food systems have not been investigated, it can be surmised from the proteolytic and surfactant nature of the treatment that application is feasible.

C.W. Donnelly: Will the developed methods be useful in improving our abilities to isolate and detect Listeria? If so, could the authors define such uses?

Authors: This enzyme/surfactant treatment would be applicable to situations in which interfering food substance could be removed leaving intact cells of L. monocytogenes. This concentrated form of cells could then be added to detection media resulting in a higher chance of recovery. This method would be particularly valuable when cell numbers of L. monocytogenes are low.

R.E. Brackett: Table 1 and Figure 4-6 are used to support the conclusion that trypsin, Triton X-100, and Tween 80 do not have an adverse effect on L. monocytogenes cells. However, data in Table 1 indicate that colony size was reduced by up to 23% (2% trypsin). Also it appears that cells shown in Figure 5 (trypsin-treated) are somewhat deformed compared to those in Figure 6. How do you explain this?

Authors: Reduction in colony size does not necessarily indicate cell size reduction; therefore, colony size was not a major factor in determining the toxicity of the chemicals.
Enzyme/Surfactant-Filtration Technique for Ice Cream

The cells in both of these micrographs were fixed directly on agar, dehydrated, impregnated with HMDS and air-dried. Comparison of cells grown at 35°C in Figures 4 (untreated), 5 (2% trypsin) and 6 (2% Tween 80) all of which were treated with HMDS, and air-dried with those in Figure 8 (2% trypsin + 2% Tween 80) which were also grown at 35°C and critical point dried suggest that size and shape variations may be due to the temperature at which the cells were cultured or to the SEM preparation. The non-deleterious effect of the trypsin treatment is supported by the Total number data illustrated in Table 1.

R.E. Brackett: The materials and methods describe a software program for measuring bacterial cell length and a statistical procedure of analyses. Are results of these analyses not presented because no significant differences were found? Also, were similar measurements done on cells shown in Figures 4-6?

Authors: The results were not presented because in actuality no significant differences were discovered. Similar statistical procedures were not performed on Figures 4-6 because visual analysis of the cells which were critical point dried (Figures 1, 7, 8, 9) in combination with the data collected in Table 1 seemed adequate to ensure no adverse effects upon the cells. Measurements were made on Figures 7-9 to investigate the possibility of decreased size or morphological changes in relation to varied temperatures and times of incubation.

J. Humber: What was the final concentration of L. monocytogenes in the ice cream mix prior to enzyme and surfactant treatment? Would the procedure have been successful when lower cell numbers are present in the ice cream mix - 10^3 cells per g, for example?

Authors: The final concentration of L. monocytogenes in the ice cream mix prior to treatment was 10^8 cells/ml. It would be possible to recover cells from samples inoculated or contaminated with low numbers of bacteria because the centrifugation and filtration following the treatment not only remove the solubilized food components but concentrate the cells as well.
LIGHT AND SCANNING ELECTRON MICROSCOPY OF THE PEANUT (Arachis hypogaea L. cv. Florunner) COTYLEDON AFTER ROASTING

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Abstract

Changes in the microstructure of peanut (Arachis hypogaea L. cv. Florunner) cotyledons after roasting at a temperature of 160°C (16 min) were investigated with light and scanning electron microscopy. Major changes included: (1) pitting and pocket-marking of the epidermis of the cotyledons caused by the escape of steam and oil released during roasting; (2) loss of cellular organization of the cytoplasmic network surrounding the lipid bodies, protein bodies, and starch grains; (3) alteration of the structures of cytoplasmic network, lipid bodies, and protein bodies; and (4) heat destruction of some middle lamellae of cell-to-cell junctions.

Introduction

The purpose of roasting peanuts is to create flavor changes that enhance the palatability of peanut products. Our peanut laboratory has been actively involved in the assessment of flavor characteristics of roasted peanut products. Young et al. (1974) investigated the effect of roasting methods on the flavor and composition of peanut butter. More recently we have evaluated the chemical changes and sensory attributes of peanuts during roasting (Oupadissakoon and Young, 1984a, b).

The purpose of the present investigation was to examine the changes in the microstructure of roasted peanuts with light and scanning electron microscopy (LM and SEM). By evaluating the microstructure of the roasted peanut, our goal was to provide information that may affect product development of roasted peanuts.

Materials and Methods

Raw peanuts (Arachis hypogaea L. cv. Florunner) were roasted for 16 min at 160°C. Both raw and roasted peanut cotyledons were then prepared for microscopic examination. Tissue blocks (1 mm³) of outer surface epidermis, mid-region parenchyma, and inner surface epidermis from both the raw and roasted peanut cotyledons were prepared for LM and SEM using the method of Young and Schadel (1989). LM specimens were photographed using a Wild photomicroscope fitted with a Wild 35mm camera. SEM specimens were viewed with a Philips 505T scanning electron microscope at a working distance of 15mm operating at an accelerating voltage of 15kV.

Results and Discussion

The embryo of the raw peanut consists of two cotyledons and a small radicle and plumule known as the "heart". The individual cotyledons contain three kinds of tissues: (1) epidermal, (2) vascular, and (3) parenchymal. The epidermis consists of a layer of cells which covers the cotyledon surface. The vascular tissue consists of one series of bundles that follow the curvature of the outer cotyledon surface and another series of bundles that are centrally located. The parenchyma makes up the greatest part of each cotyledon with comparatively large, almost isodiametric cells. The major subcellular organelles of these cells in the resting peanut cotyledon are lipid bodies (oil reserve bodies), aleurone grains (protein bodies), and starch grains.

Key Words: Light microscopy, scanning electron microscopy, microstructure, roasted peanuts.

*Paper number 11732 of the Journal Series of the North Carolina Agriculture Research Service, Raleigh, NC. The use of trade names in this publication does not imply endorsement by North Carolina Agriculture Research Service of the products named, nor criticism of similar ones not mentioned.
Fig. 1. Scanning electron micrograph of the epidermal cells of the rounded, outer surface of a raw peanut cotyledon. Bar = 15 micrometers.

Fig. 2. Scanning electron micrograph of the epidermal cells of the rounded, outer surface of a roasted peanut cotyledon. Note the presence of pitting and pock-marking (arrows) which results from the escape of steam and oil released during roasting. Bar = 15 micrometers.

Fig. 3. Scanning electron micrograph of the epidermal cells of the flat inner surface of a raw peanut cotyledon. Bar = 15 micrometers.

Fig. 4. Scanning electron micrograph of the flat, inner surface of a roasted peanut cotyledon. Extruded oil (arrows) is present on the surface of the cells. Bar = 15 micrometers.

Fig. 5. Scanning electron micrograph of a cell wall (w) and the cytoplasmic network (c) surrounding the storage reserve bodies of lipid, protein, and starch in a raw peanut cotyledon. Bar = 3 micrometers.
This study revealed that roasting the intact peanut embryos affected all tissues and almost all subcellular organelles via thermal modification. Beginning with the epidermal cells of the rounded outer surface of a raw cotyledon (Fig. 1), we observed pitting and pock-marking of the epidermis which resulted from the escape of steam and oil released during roasting (Fig. 2). The flat, inner surface of the resting seed cotyledon (Fig. 3) was also characterized by the presence of oil released after roasting (Fig. 4).

In the raw peanut cotyledon the cytoplasmic network surrounds the major storage reserve bodies of protein, lipid, and starch (Figs. 5 and 6). After roasting there is a loss of this cellular organization of cytoplasmic network. This loss of organization is a result of thermal modification (Figs. 7 and 8). Cytoplasmic compartmentalization of the oil is lost in the majority of the cells as the oil becomes heated and escapes. During roasting, protein bodies distend (Fig. 8) as heat turns the small amount of water present in the cells into steam. The starch grains...
However, remain relatively unaltered by this steam which is insufficient to gelatinize the individual starch grains (Fig. 8).

In the raw peanut cotyledon, the cells of the rounded outer surface are not swollen when observed in transverse section (Fig. 9). After roasting these cells become slightly swollen as heat destroys the structure of cytoplasmic network and distends protein bodies (Fig. 10 and 11).

In the raw peanut cotyledon the cells of the flat, inner surface (Fig. 12) also have a typical well-organized appearance of cytoplasmic network surrounding the lipid bodies, protein bodies, and starch grains. After roasting, the thermal modification of these cells (Figs. 13 and 14) is not as prominent as in the outer surface cells possibly because of a lag in conduction of heat from the outer surface to the inner surface. The conduction of heat from the outer surface of the cotyledon to the inner surface is sufficient, however, to destroy some of the middle lamellae of cell-to-cell junctions (Fig. 14), although less protein body distension was observed in the inner cell surfaces.
Physical Structure of Roasted Peanuts

All of the changes we observed in the micro-structure of peanuts after roasting affect the taste characteristics of the roasted product; these, in turn, determine the usefulness of the peanuts in peanut butter, confections, and other food items. Further microscopical investigations of peanuts roasted under different conditions coupled with evaluation of sensory attributes should provide valuable information for processors who wish to enhance the taste characteristics of roasted peanut products.

References


Discussion with Reviewers

R. Taber: Figs. 9 and 10 contrast the unroasted versus the roasted peanut. Fig. 9 depicts cells that are cut through to expose the interior of the cells. The interpretation of the "swollen cells" in Fig. 10 is fairly straight-forward; however, none of the interior parts of the cells appear to be exposed. What was the difference in their preparation and can they actually be compared?

Authors: The samples for the raw peanut cellular microstructure depicted in Fig. 9 and the roasted peanut cellular microstructure depicted in Fig. 10 were prepared using identical SEM preparation procedures. The loss of structural details in the roasted peanut cells may be due to a complication caused by the presence of decompartmentalized oil. With regard to this matter, W.J. Wolf (personal communication) has suggested that hexane could be used to defat the specimens. If the material obscuring structural detail was decompartmentalised oil, the hexane would remove the oil but not dissolve the protein or carbohydrate. This would enable a better comparison between protein bodies and starch grains in raw and roasted peanut cells.

Legends for Figures on the facing page

Fig. 10. Scanning electron micrograph of a transverse section of the cells of the rounded, outer surface of a roasted peanut cotyledon. Note that heat has destroyed the cytoplasmic network and obscured structural details; cell wall (w) is most recognisable structure. Bar = 5 micrometers.

Fig. 11. Light micrograph of a transverse section of the cells of the rounded, outer surface of a roasted peanut cotyledon. Note that the swollen cellular contents have broken an outer cell wall (w). Bar = 5 micrometers.

Fig. 12. Scanning electron micrograph of a transverse section of cells of the flat, inner surface of a raw peanut cotyledon. Note the typical well-organized appearance of cytoplasmic network (c). Bar = 10 micrometers.

Fig. 13. Scanning electron micrograph of a transverse section of the cells of the flat, inner surface of a roasted peanut cotyledon. Bar = 10 micrometers.

Fig. 14. Light micrograph of a transverse section of the cells of the flat, inner surface of a roasted peanut cotyledon. Note that the heat is sufficient to destroy some of the middle lamellae of cell-to-cell junctions (arrows) which in turn increases the size of intracellular spaces after roasting. Bar = 5 micrometers.
TECHNICAL NOTE:

A SIMPLE CARRIER FOR FREEZING DIFFICULT FOOD SAMPLES
IN PREPARATION FOR SCANNING ELECTRON MICROSCOPY

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Abstract

A thin aluminum foil carrier allows several pre-shaped samples to be plunge-frozen simultaneously. The disposable, inexpensive, simple-to-make carrier allows effective freeze-fracture electron microscopy of troublesome samples.

Introduction

Successful scanning electron microscopy (SEM) of freeze-fractured samples having a low total solids content such as yoghurt or renneted milk curd occasionally requires extraordinary treatment (1, 3); these samples remain to be very soft even after prolonged glutaraldehyde fixation. Treatments usually involve mixing the samples with agar sol (4) or encapsulation in agar gel tubes (2); both disrupt sample structure and are suitable only to study spreadable viscous foods. Agar becomes integrated into the sample structure in the former case, whereas encapsulation requires aspiration and extrusion of the sample into tubes, which disturbs the initial structure. The best preparation for difficult samples is the one that minimizes handling and disruption of structure.

Cracking and breaking of soft samples during handling with forceps is particularly troublesome during plunge-freezing; submerging the sample in the cryogen and holding if there is difficult, but was made possible by the use of a simple, disposable aluminum foil carrier.

Materials and Methods

The carrier, made from thin (0.01 mm thick) aluminum foil (Fisher Scientific, Nepean, Ontario) allowed the maximum sample cooling rate during plunge-freezing. Three selected folds of a 10 mm x 25 mm foil piece formed a pocket to contain the samples (Fig. 1a-c); the dull side faced inwards to reduce sample adhesion to the carrier.

Samples, fixed in a glutaraldehyde solution and dehydrated in ethanol were loaded into the carrier and a few drops of ethanol were added to keep the samples wet (Fig. 1d). The carrier was closed (Fig. 1e) and the assembly was gripped using forceps (Fig. 1f), plunged into a melted pool of Freon 12 at its freezing temperature, and submerged for 15-20 s. Then the carrier was transferred into liquid nitrogen and the sample was taken out (Fig. 1g). The samples were fractured as usual (Fig. 1h). Standard transferral of fractured particles to the critical point drying basket and subsequent particle handling followed (Fig. 1i).

Results and Discussion

Advantages of using the aluminum foil carrier over direct specimen handling using forceps include: increased efficiency when carriers are made ahead of time and are used to prepare multiple samples simultaneously (Fig. 1h) and reduction of damage when preparing difficult samples. Pre-shaping of samples further increases the efficiency and success of the technique. If samples are pre-shaped (cut
into sticks 15 mm long with a square face of 1 mm per side) before glutaraldehyde fixation and processing for SEM, the only further manipulation of the sample required is at the freeze-fracture step. After critical point-drying, the dried samples may be mounted on stubs without any further trimming. Trials were carried out where samples including yoghurts, milk gels, and cheeses were trimmed later in the processing schedule. Some samples were found to be more difficult to handle; after glutaraldehyde fixation and/or dehydration in ethanol, cheeses became very tough and brittle, and, in some cases, shattered when cut. Cheese samples were tougher and more brittle when trimming was delayed until after critical point drying. Yoghurts and milk gel samples became firmer and were slightly easier to handle without damage if cut later in the processing schedule. When trimmed after critical point-drying, they sometimes broke into small, useless fragments. It is therefore recommended that all samples be pre-shaped before processing. The benefits of adopting this practice include better penetration of chemicals into the samples, and reduction of the exposure of the microscopist to chemicals compared to the situation when large samples are trimmed in the later stages of the processing schedule.

Pre-shaping of the samples allows precise control of fracture location; this is important when samples contain some structural orientation (e.g., cheddared curd or stretched Mozzarella cheese) and fracturing in more than one orientation is desired. Careful alignment of the samples during carrier loading allows more efficient fracturing by having each blade stroke fall across all aligned pieces simultaneously. Straight fractures are effected, saving more time during mounting and electron microscopy of fracture faces.

The carriers have been used in the author’s laboratory for a wide range of dairy food samples, and have been found to be very versatile. They are also useful in the preparation of routine samples, where they allow for rapid preparation for the SEM.

Acknowledgment

Contribution 850 from the Food Research Centre.

References

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The peanut cotyledon cellular structure is highly organized containing oil, protein, and starch. This scanning electron micrograph shows the cellular organization of the cells of the rounded outer surface of a peanut cotyledon before roasting. The effect of roasting on peanut cotyledon microstructure is examined in a paper by C. T. Young and W. E. Schadel (pages 69-73).